学 位 論 文

The effects of maternal polyphenol intake during lactation
on lipid metabolism in adult rat offspring
(授乳期母親のポリフェノール摂取が成熟期仔ラットの脂質代謝に及ぼす影響)

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Contents

CHAPTER 2

The protective effects of maternal green tea extract intake during lactation against hepatic lipid accumulation in adult male rats exposed to a continuous high-fat diet from the foetal period

ABBREVIATIONS

8-OHdG: 8-hydroxy-2'-deoxyguanosine, ACC: Acetyl-CoA carboxylase, AMPK: AMP-activated protein kinase, apo: Apolipoprotein, BMI: Body mass index, BUN: Blood urea nitrogen, CON: Control diet, CVDs: Cardiovascular diseases, CYP7A1: Cholesterol 7 alpha-hydroxylase, DGAT: Diglyceride acyltransferase, dl: Deciliter, DM: Diabetes mellitus, EGCG: Epigallocatechin-3-gallate, ER: Endoplasmic reticulum, FAS: Fatty acid synthase, Fig: Figure, GTE: Green tea extract, HF: High fat, H&E: Hematoxylin and eosin, HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase, Insig: Insulin induced gene protein, kg: Kilogram, kΩ: Kilo ohm, LCAD: long-chain acyl-CoA dehydrogenase, LDL: low-density lipoprotein, mg: Milligram, min: Minute, MTTP: Microsomal triglyceride transfer protein, NAFLD: Non-alcoholic fatty liver disease, NCDs: Non-communicable diseases, NASH: Non-alcoholic steatohepatitis, PAGE; Polyacrylamide gel electrophoresis, PPARα: Peroxisome proliferator-activated receptor α, RSV: Resveratrol, S1P: Site-1 protease, S2P: Site-2 protease, Sirt1: Sirtuin 1, SE: Standard error, SOAT: Sterol O-acyltransferase, SREBP: Sterol regulatory element-binding protein, TG: Triglyceride, v: Volume, VLDL: Very low-density lipoprotein, WHO: World Health Organization

PREFACE

Lipid metabolic disorders

Lipids, along with carbohydrates and proteins, are irreplaceable nutrients for humans. In the human body, various lipids including triglyceride (TG), fatty acid (FA), and cholesterol exist and play crucial roles such as storing energy, signaling, and acting as structural components of cell membranes (Fahy et al. 2009; Subramaniam et al. 2011). Lipid is strictly controlled its homeostasis in the body due to the importance and variety of its function. Lipid homeostasis imbalance, in other words, lipid metabolic disorders are often found as obesity, overweight, hyperlipidemia, and non-alcoholic fatty liver diseases (NAFLD). For adults, World Health Organization (WHO) defines overweight is a body mass index (BMI) greater than or equal to 25 and obesity is a BMI greater than or equal to 30. And WHO estimated that 39% of the world's adult (18 years and older) population is overweight and 13% is obese in 2016, meaning more than 1.9 billion adults are overweight, of whom 650 million are obese. And prevalence of obesity nearly tripled between 1975 and 2016 (WHO 2020). NAFLD exists on a spectrum from simple fatty liver to an inflamed fatty liver (non-alcoholic steatohepatitis; NASH) (Ludwig et al. 1980; Schaffner & Thaler 1986; Younossi et al. 1998). The global prevalence of NAFLD in the general population has been estimated to be 25% and the global prevalence of NASH has

been estimated to range from 3% to 5% (Vernon et al. 2011; Younossi et al. 2016; Younossi et al. 2018). These data indicate that lipid metabolic disorders are now globally common diseases.

Studies have indicated that lipid metabolic disorders are risk factors for non-communicable diseases (NCDs) and can progress to severe diseases. The main types of NCDs are cardiovascular diseases (CVDs), cancers, chronic respiratory diseases and diabetes mellitus (DM). Currently, NCDs are one of the most serious health issues. WHO estimated that NCDs caused 70% of all deaths in 2016 (WHO 2018). Studies have reported a relationship between hypercholesterolemia and deaths caused by CVDs, indicating that hypercholesterolemia is a risk factor of CVDs (Kannel et al. 1986; Chen et al. 1991; Stamler et al. 2000; Emberson et al. 2003; Grau et al. 2010; Imano et al. 2011; Sugiyama et al. 2015). NAFLD, especially NASH is reported that it can progress to more serious disease stages, such as advanced fibrosis, cirrhosis, liver failure, or liver cancer (Powell et al. 1990; Angulo 2002; Vernon et al. 2011; Torres et al. 2012; McPherson et al. 2015). Global age-standardized prevalence in 2017 was estimated 11.061 cases per 100 000 for cirrhosis due to NASH and 1.20 cases per 100 000 for liver cancer due to NASH (James et al. 2018). Moreover, studies have reported the close relationship between obesity or overweight and DM and its risk factor, insulin resistance

(Wagenknecht et al. 2003; Kahn et al. 2006).

Factors of lipid metabolic disorders

As a risk factor of lipid metabolic disorders and NCDs, theories called Fetal Origins of Adult diseases (FOAD) and Developmental Origins of Health and Diseases (DOHaD) describe that NCDs can be induced by environmental factors in early life such as prenatal and infancy. FOAD is based on epidemiological studies in Britain in the 20th century. It was found that low birth weight, in other words, poor fetal nutrition, was a major contributor to CVDs mortality (Barker 1990). And hypothesis was stated that prenatal undernutrition permanently changes the body's structure, function, and metabolism in ways that lead to CVDs in later life (Barker 2007). After FOAD, researchers have developed DOHaD concept that not only the prenatal nutritional status but also environmental factors from prenatal to infancy affect the future health of children via alteration of the development in early life. The part of these concepts that poor nutritional condition during development increases the future risk of diseases is supported by a large number of studies including studies on future health impacts by Great Famine. And it has also been reported long-term effects on disease risk associated with birth weight within the normal range in addition to extremely low body weight in

epidemiological studies (Hanson & Gluckman 2014). Furthermore, both epidemiological and animal studies have shown that the DOHaD concept operates not only in poor nutritional environment, but also in excessive or rich nutritional environment (Armitage et al. 2005; Catalano & Ehrenberg 2006; Reynolds et al. 2013; Ribaroff et al. 2017; Gomes et al. 2018). Another study showed that maternal high fat (HF) diet intake induces sustained alterations of predispositions to develop obesity and addictive-like behaviors across multiple generations in the absence of any further exposure to HF diet (Sarker et al. 2018). The prevalence of overweight and obesity in women in 2016 is estimated at approximately 40% and 15%, respectively (WHO 2020). Obesity should be concerned as the problem similar to the problem of "diabetes begetting diabetes", by which gestational DM strongly induces DM in children (Ma & Chan 2009; Yajnik 2010).

Prevention of lipid metabolic disorders

From the view of DOHaD, avoiding maternal overnutrition during gestation and lactation is crucial to prevent lipid metabolic disorders in children. In this respect, polyphenol supplementation for mothers during developmental period is expected to decrease future risk of diseases in children. Polyphenol is known to have various beneficial physiological effects for humans. Studies have reported its antioxidant, anti-

inflammatory, anti-cancer properties. Moreover, polyphenol can regulate energy metabolism, in other words, prevent energy metabolic disorders (Bravo 2009; Brookins Danz et al. 2009; Crozier et al. 2009; Dai & Mumper 2010; Mahmoodi et al. 2020; Musial et al. 2020). It can be effective on children directly via breast milk (Franke et al. 2006; Romaszko et al. 2014; Khymenets et al. 2016), and indirectly by avoiding metabolic disorders in mothers that affect children.

Previous studies have reported the beneficial effects of maternal polyphenol intake. Maternal resveratrol (RSV) intake added to HF diet during pregnancy and lactation decreased body weight and adipose tissue content in offspring (Ros et al. 2018). Maternal green tea extract (GTE) intake is expected to have protective effects against diet-induced diseases in children as shown in researches (Sato et al. 2013; Hachul et al. 2018; Kataoka et al. 2018). Moreover, maternal polyphenol intake may be effective treatment to prevent diet-induced diseases in children. Li et al. (2012) reported that maternal GTE intake added to HF diet improved metabolic disorders in rat offspring more than GTE intake by the offspring itself after weaning. Ehara et al. (2012) suggested the change in hepatic gene expression in response to nutritional demand during development is affected by maternal nutritional status. This implies that the effects of maternal polyphenol intake on offspring are altered depending on the step of development. Indeed,

several studies showed the impacts of maternal nutrition on offspring can be influenced by the duration of exposure to it (Gregorio et al. 2010; Wang et al. 2012; Sarker et al. 2019). There have been studies conducted to examine effects of maternal polyphenol intake during development on offspring, however, limited studies were conducted to examine the effects of maternal polyphenol intake during lactation on lipid metabolism in adult offspring.

Objective of the thesis

This thesis is aimed to examine the effects of maternal polyphenol intake during lactation on lipid metabolism in adult male offspring and its underlying mechanisms.

Structure of this thesis

This thesis consists of two chapters.

Chapter 1: The effects of maternal resveratrol intake during lactation on cholesterol metabolism in adult male rat offspring.

RSV is a kind of polyphenol that has various beneficial physiological activity and it is expected to have protective effects against NCDs by improving lipid metabolism. Maternal nutrition environment during the developmental period, including lactation,

affects the future risk of NCDs in children. Several studies to report the effects of maternal RSV intake on offspring, but few studies were conducted to examine the effects of maternal RSV intake during lactation on lipid metabolism in adult offspring. In this study, I focused on the effects of maternal RSV intake during lactation on cholesterol metabolism in adult rat offspring. Moreover, I examined changes in hepatic lipid metabolism of the offspring to evaluate the underlying mechanisms.

Chapter 2: The protective effects of maternal green tea extract intake during lactation against hepatic lipid accumulation in adult male rats exposed to a continuous high-fat diet from the foetal period.

GTE which contains rich polyphenol such as EGCG, has various beneficial physiological activities to improve and prevent NCDs and lipid metabolic disorders. Studies have reported that maternal overnutrition during developmental period can induce future lipid metabolic disorders like NAFLD in offspring. In this study, I focused on the effects of maternal GTE intake on lipid metabolism, particularly hepatic lipid accumulation, in adult rat offspring exposed to continuous high-fat diet from foetal period. Moreover, I examined changes in hepatic lipid metabolism of the offspring to evaluate the underlying mechanisms.

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CHAPTER 1

Long-term effects of maternal resveratrol intake during lactation on cholesterol metabolism in male rat offspring

Abstract

Background: Maternal nutrition status during developmental period, including lactation affects future risk of diseases in children. Resveratrol (RSV) can protect against diseases by improving lipid metabolism. However, it is unclear that effects of maternal RSV intake on health of adult offspring.

Objective: The aim of this study was to examine the effects of maternal RSV intake during lactation on lipid metabolism particularly cholesterol in adult male rat offspring.

Materials and methods: Female Wistar rats were fed a control diet (CON) supplemented with or without RSV (20 mg/kg body weight/day) during lactation. Male offspring were weaned onto CON and were divided into two groups (CON/CON, CON+RSV/CON) until 36 weeks.

Results: A significant plasma cholesterol level significantly was lower in RSV-offspring compared to CON-offspring, while no significant change was found in levels of hepatic cholesterol and triglyceride between the two groups. A significant lower level of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase and a significant higher level of hepatic LDL-receptor were observed in the RSV-offspring. A significant lower rate of mature/precursor sterol regulatory element-binding protein 2 level was observed. And significant higher insulin-induced gene protein 1 level was found in RSV-offspring.

Conclusion: It is showed that maternal RSV intake during lactation with normal diet attenuates plasma lipid level in adult rat offspring. As the underlying mechanism, suppression of hepatic lipid biosynthesis and promotion of lipid endocytosis are suggested.

Key words: Resveratrol; maternal supplements; adult offspring; cholesterol

Introduction

It is a serious issue that the globally increased number of deaths due to non-communicable diseases (NCDs) especially cardiovascular diseases (CVDs). World Health Organization (WHO) (2018) reported that the annual number of deaths from CVDs increased by more than 3 million between 2000 and 2016. Several studies have reported a relationship between hypercholesterolemia and deaths caused by CVDs, indicating that hypercholesterolemia is a risk factor of CVDs (Kannel et al. 1986; Chen et al. 1991; Stamler et al. 2000; Emberson et al. 2003; Grau et al. 2010; Imano et al. 2011; Sugiyama et al. 2015). Since cholesterol is an essential lipid found in hormones, bile, and the cell membrane, its homeostasis in the body is strictly regulated. However, excess cholesterol is known to be a risk factor for CVDs as indicated in the above-mentioned studies.

Hepatic cholesterol metabolism exhibits a close relationship with cholesterol homeostasis in the body since the liver is the main organ that regulates cholesterol homeostasis. In the liver, cholesterol homeostasis is controlled by four main routes: biosynthesis, uptake from blood, supply into blood, and conversion to bile acid. SREBP-2, a key regulator of cholesterol metabolism, is retained on the endoplasmic reticulum (ER) by its association with insulin-induced gene protein (Insig) in the presence of

cholesterol. However, as the intracellular cholesterol level decreases, SREBP-2 is released from the ER and is transported to the Golgi apparatus (Yang et al. 2002). Furthermore, upon cleavage by the two proteases, Site-1 protease (S1P) and Site-2 protease (S2P), in the Golgi, the N-terminus of SREBP-2 which has transcriptional activity is transferred to the nucleus (Sakai et al. 1996; Duncan et al. 1997). SREBP-2 regulates the transcription of cholesterol metabolism-related proteins, such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and LDL-receptor (Horton et al. 1998; Sakakura et al. 2001). Cholesterol is synthesised from acetyl-CoA. HMGCR is the enzyme that determines the rate of cholesterol biosynthesis (Geelen et al. 1986; Goldstein & Brown 1990). Statins, widely used as therapeutic agents for CVDs, reduce serum cholesterol levels by inhibition HMGCR activity (Brown et al. 1978; Reihnér et al. 1990; Goldstein & Brown 2015). LDL-receptor plays important role in lipid uptake (Brown & Goldstein 1983; Spady 1992). LDL-receptor translocates lipid-rich particles, LDL and very low-density lipoprotein (VLDL), and chylomicron remnant from the blood into cells (Mahley 1988; De Faria et al. 1996; Williams 2008). In the pathway of cholesterol supply into blood, hepatic sterol O-acyltransferase (SOAT)-2 and microsomal triglyceride transfer protein (MTTP) are important. SOAT-2 esterifies free cholesterol in hepatic lipid droplets with acyl-CoA (Chang et al. 2009; Marshall et al. 2014), while MTTP associates

triglyceride (TG) and cholesterol ester with apo-B protein to form VLDL (Gordon & Jamil 2000; Hussain et al. 2012). To convert cholesterol into bile acid, cholesterol 7 alpha-hydroxylase (CYP7A1) is a crucial. CYP7A1 is an enzyme involved in the “classical pathway," which is the major pathway for bile acid synthesis and one of the few cholesterol excretion pathways (Chiang 2009).

It is already known that personal lifestyles, such as excessive nutritional intake or less exercise, play an essential role in dyslipidemia and the development of NCDs. On the other hand, recent studies have revealed that maternal nutrition during pregnancy and lactation affects the future health of children. Both epidemiological and animal studies have shown that maternal malnutrition or overnutrition increase the risk of NCDs in children. Epidemiological studies showed that fetal exposure to famine increases the risk of hyperglycemia in adulthood (Ravelli et al. 1998; Li et al. 2010). Animal studies have reported that maternal high-fat diet could lead to obesity, insulin resistance, and hyperlipidemia, including hypercholestrolemia, in their offspring (Samuelsson et al. 2008; Ribaroff et al. 2017). Furthermore, it has been suggested that the consumption of high-fat diet by mothers during lactation is more responsible in affecting lipid metabolism in offspring than the consumption of such a diet during pregnancy (Gregorio et al. 2010; Sun et al. 2012; Sarker et al. 2019). In other studies, the effects of long-term maternal

treatment with peroxisome proliferator-activated receptor α (PPARα) ligand during lactation were examined in mouse offspring (Ehara et al. 2015; Yuan et al. 2018).

Resveratrol (3,5,4-trihydroxystilbene; RSV) is a type of plant polyphenol found in red wine and grapes, and is known for its antioxidant, anti-inflammatory, and anti-cancer properties (Brookins Danz et al. 2009). Furthermore, its preventive effect against diet-induced dyslipidemia has also been reported (Cho et al. 2008; Zhu et al. 2008). These biological effects of RSV depend on the activation of Sirtuin 1 (Sirt1) (Lagouge et al. 2006; Brookins Danz et al. 2009) which regulates the activation of AMP-activated protein kinase (AMPK) via deacetylation and activation of Liver Kinase B1 (Hou et al. 2008; Lan et al. 2008). Studies have already reported that activated AMPK attenuates hepatic cholesterol biosynthesis (Henin et al. 1995; Liu et al. 2015), and along with RSV affects cholesterol metabolism. Li et al. (2011) reported that RSV attenuated hepatic cholesterol biosynthesis by the activation of AMPK. In addition, other studies have indicated that RSV could ameliorate hypercholesterolemia (Do et al. 2008; Xin et al. 2013; Yu et al. 2016).

Several studies have examined the effects of maternal RSV intake in offspring. Our previous study (Tanaka et al. 2017) reported the long-term effects of maternal RSV intake during the lactation on lipid metabolism in adult rat offspring. The study has shown

attenuated plasma TG content in adult rat offspring. As the mechanism, it was showed suppressed hepatic lipogenesis: lower level of fatty acid synthase and acetyl-CoA carboxylase, and inactivated SREBP-1. It was also found that RSV activated Sirt1, one of histone deacetylases, and AMPK in offspring liver. Those suggest that maternal RSV intake affects lipogenesis via epigenetic alterations, which is heritable changes in gene function that take place without a change in the DNA sequence. However, studies examining cholesterol metabolism with respect to RSV intake are limited. Ros et al. (2018) reported that maternal RSV treatment with high-fat or low-fat diet during pregnancy and lactation did not cause significant changes in serum cholesterol level in rat offspring on postnatal day 0 and 21. Similarly, Vega et al. (2016) showed that RSV exposure during gestation did not significantly affect the serum cholesterol level in both male and female rat offspring on postnatal day 110. On the other hands, Sun et al. (2019) reported that maternal RSV intake during pregnancy and lactation increased HDL-cholesterol and LDL-cholesterol level in piglet offspring on postnatal day 21. The influence of maternal RSV intake on cholesterol metabolism in offspring cannot be concluded, because these studies were conducted under different experimental conditions and did not examine the underlying mechanisms in detail. Therefore, further investigations are needed to elucidate the effects of maternal RSV supplementation on

cholesterol metabolism in the offspring.

Objective

This study was aimed to examine the effects of maternal RSV ingestion during lactation on cholesterol metabolism in rat offspring and its underlying mechanism.

Materials and Methods

Animal treatments

All procedures were performed according to the Guidelines for Animal Experimentation, Aomori University of Health and Welfare. Pregnant Wistar rats were divided into two dietary groups and were fed a control diet (CON; MF diet; Oriental Yeast, Tokyo, Japan) during gestation and CON or CON supplemented with RSV (Sigma-Aldrich, Tokyo, Japan) during their lactation. The mother rats in the CON supplemented with RSV group received RSV solution orally (20 mg/kg body weight) once a day by gavage during lactation, while the mother rats in the other group received a vehicle (0.05% carboxymethylcellulose) orally once a day by gavage during lactation. Six male offspring of each group were examined for this study. At 3 weeks of age, the male offspring were weaned onto CON and maintained on this diet for 36 weeks. The body weights and food intake of each offspring were recorded after weaning every 4 weeks. At 36 weeks, the offspring were fasted overnight and killed under ether anesthesia (Figure 1). Their blood and livers were immediately extracted, and the liver samples were stored at -80℃ before further evaluation.

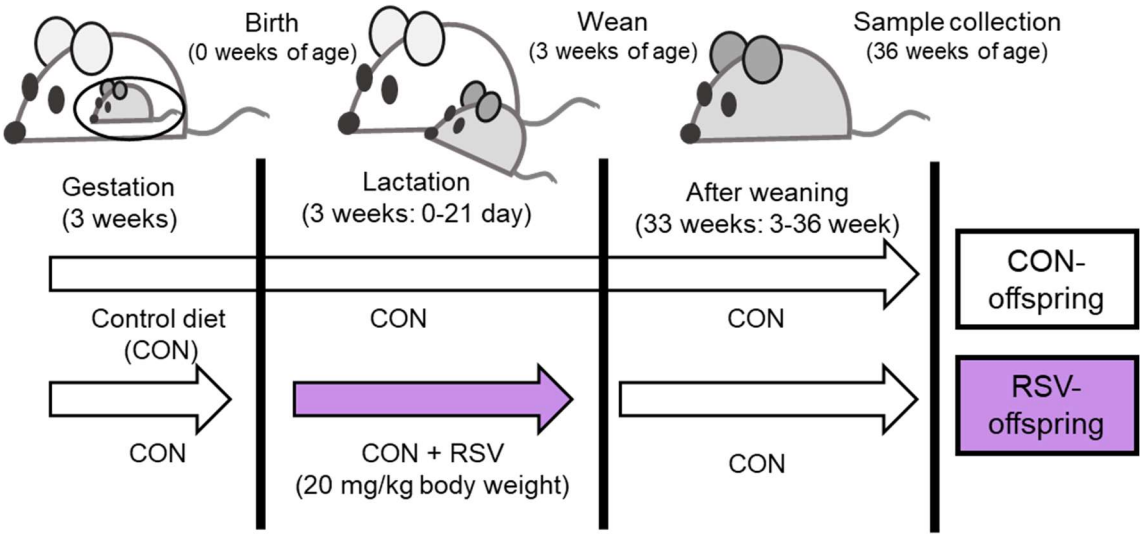

Figure 1. Experimental design.

CON offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV offspring: a control diet during gestation, 20 mg/body weight RSV added once a day in the control diet during lactation, and a standard diet after weaning.

Blood chemistry analysis

Plasma samples were obtained after centrifugation and examined for levels of total cholesterol, blood glucose, blood urea nitrogen (BUN), and creatinine using an autoanalyser for blood chemistry analysis (Fuji Dri-Chem 3500 V; Fuji Film, Tokyo, Japan).

Hepatic lipid level

The liver sample was added to a solution of chloroform and ethanol (1:2, v/v) in an Eppendorf tube and homogenized. After incubation, chloroform was added to the

mixture and blended. Next, distilled water was added and mixed again. The chloroform layer was separated from the samples after centrifugation. The lipid fraction was obtained by evaporating chloroform with an evaporator and was dissolved in isopropanol. TG and total cholesterol levels in the sample were measured using LabAssay Triglyceride Kit and LabAssay Cholesterol Kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) according to the manufacturer's instructions.

Western blot analysis

For western blot analysis, the liver samples were homogenized in a buffer using a Polytron (PCU Drehzahlregler; Kinematica, Luzern, Switzerland) on ice. The homogenate was centrifuged at 4℃, and the supernatant was collected. Next, the obtained supernatant was heated to avoid denaturation of protein and the protein concentration in the sample was measured by Bradford assay (Protein Assay; BIO-RAD, Hercules, U.S.A.) (Bradford 1976). Proteins in the sample were separated by SDS-PAGE by using biotinylated protein molecular weight markers (M&S TechnoSystems, Inc., Osaka, Japan) as protein standards. Proteins were then electrophoretically transferred onto a nitrocellulose membrane using the iBlot transfer system (Thermo Fisher Scientific K.K., Tokyo, Japan). The nitrocellulose membrane was incubated overnight at 4℃ in a blocking

solution containing 3% skim milk. The membrane was then washed and exposed to primary antibodies: CYP7A1 (bs-2399R; Bioss, Massachusetts, U.S.A.), HMGCR (ab180615, Abcam, Tokyo, Japan), Insig-1 (ab70784; Abcam, Tokyo, Japan), MTTP (ab186446, Abcam, Tokyo, Japan), LDL-receptor (3839-30T; BioVision, California, U.S.A.), S1P (ab140592, Abcam, Tokyo, Japan), S2P (ab140594; Abcam, Tokyo, Japan), SREBP-2 (ab30682, Abcam, Tokyo, Japan), SOAT-2 (bs5020R; Bioss, Massachusetts, U.S.A.), and beta-Actin (ab8226, Abcam, Tokyo, Japan), in the presence of a 1% blocking solution. Next, the membrane was again washed and exposed to the secondary antibodies: anti-rabbit IgG IRDye 680 (926-68071; M&S TechnoSystems, Inc., Osaka, Japan) or anti-mouse IgG IRDye 800 (926-3221; M&S TechnoSystems, Inc., Osaka, Japan), in presence of a 1% blocking solution. Protein bands were quantitated using Odyssey infrared imaging system (M&S TechnoSystems, Inc., Osaka, Japan). Protein levels were normalized against those of beta-actin from the same sample.

Statistical analysis

Each value was expressed as mean ± SEM. Statistical analyses were performed using Student's t-test. In all cases, $p<0.05$ was considered as statistically significant.

Results

Body weight, food intake, blood chemistry parameters

Although the dietary RSV intake did not show any significant difference between the two groups, it was observed that the body weight of RSV offspring was significantly lower than that of the CON offspring from the age of 8 weeks to 32 weeks (Table 1 and Table 2). No significant change was observed in levels of blood glucose, BUN, and creatinine between the two dietary groups at 36 weeks (Table 3).

Table 1. Body weight of male rat offspring after weaning from 4 weeks to 36 weeks.

Weeks	4	8	16	24	32	36 †
CON-offspring	148.83 ± 4.25	407.97 ± 8.92	623.0 ± 23.87	721.25 ± 36.58	798.25 ± 49.51	814.42 ± 57.73
RSV-offspring	126.55 ± 8.49	354.95 ± 7.31*	532.85 ± 7.89*	597.12 ± 16.94*	652.65 ± 26.32*	657.73 ± 28.9*

CON-offspring: a control diet during gestation and lactation, and a standard diet after weaning. RSV-offspring: a control diet during gestation, 20 mg/body weight RSV added once a day in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared to CON offspring. † At sacrifice.

Table 2. Relative food intake (g/100g body weight) of male rat offspring after weaning from 4 weeks to 32 weeks.

Weeks	8	16	24	32
CON-offspring	7.10 ± 0.12	4.61 ± 0.15	3.68 ± 0.13	3.39 ± 0.14
RSV-offspring	7.67 ± 0.30	4.77 ± 0.14	3.99 ± 0.14	3.81 ± 0.14

CON-offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV-offspring: a control diet during gestation, 20 mg/body weight RSV added once a day in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean ± SEM (n = 6).

Table 3. Blood chemistry parameters in male rat offspring at 36 weeks: plasma glucose, BUN, and creatinine.

Parameter	Glucose	BUN	Creatinine
CON-offspring	161.15 ± 6.44	17.00 ± 0.62	1.03 ± 0.05
RSV-offspring	150.17 ±2.63	15.62±0.85	1.07 ± 0.03

CON-offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV-offspring: a control diet during gestation, 20 mg/body weight RSV added once a day in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean ± SEM (n = 6).

Lipids level in liver and plasma

There was no significant difference in the levels of hepatic TG and cholesterol between the two groups (Figure 2(A) and (B)). However, plasma cholesterol level in the RSV offspring was shown to be significantly lower than that in the CON offspring (Figure 2(C)). These results suggested that maternal RSV ingestion alters cholesterol metabolism in offspring.

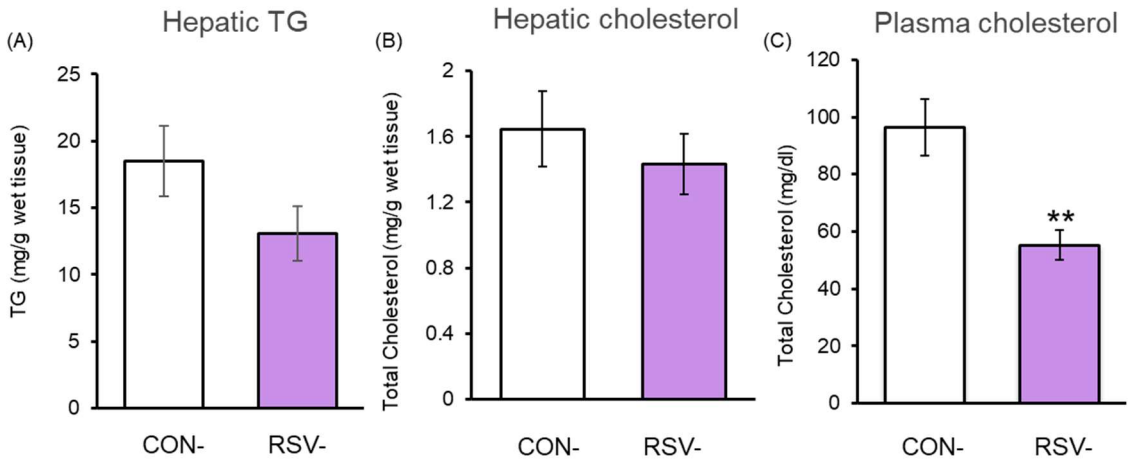

Figure 2. Hepatic TG and cholesterol content (mg/g wet tissue) (A and B) and plasma cholesterol level (mg/dl) (C) in male rat offspring at 36 weeks.

The left column indicates CON offspring, and the right column indicates RSV offspring. CON offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV offspring: a control diet during gestation, 20 mg/body weight RSV added once a day in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean ± SEM (n = 6). ** $p < 0.01$ compared to CON offspring.

Hepatic cholesterol metabolism

Cholesterol synthesis; the protein level of HMGCR

Hepatic cholesterol metabolism was investigated as a factor that affects cholesterol localization between liver and blood. During this investigation on hepatic cholesterol biosynthesis, a lower level of hepatic HMGCR was observed in RSV offspring compared with that in CON offspring (Figure 3(A)).

Lipid uptake; the protein level of LDL-receptor

Hepatic low-density lipoprotein (LDL)-receptor level was measured to evaluate hepatic lipids uptake from the blood. In this study, a higher level of hepatic LDL-receptor was observed in RSV offspring compared to CON offspring (Figure 3(B)).

Cholesterol supply into blood; the protein levels of SOAT-2 and MTTP

Hepatic SOAT-2 and microsomal MTTP levels were measured to investigate changes in the cholesterol transfer from liver to blood. In this study, there was no significant difference observed between the two groups in the levels of both SOAT-2 and MTTP, suggesting that there was no change in cholesterol supply from the liver to the blood (Figure 3(C) and Figure 3(D)).

Cholesterol excretion; the protein level of CYP7A1

To analyse cholesterol catabolizing pathways, hepatic CYP7A1 level was measured. In this study, no significant difference in hepatic CYP7A1 level was found

between the two groups, which suggested that there was no change in the bile acid synthesis through the cholesterol excretion pathway (Figure 3(E)).

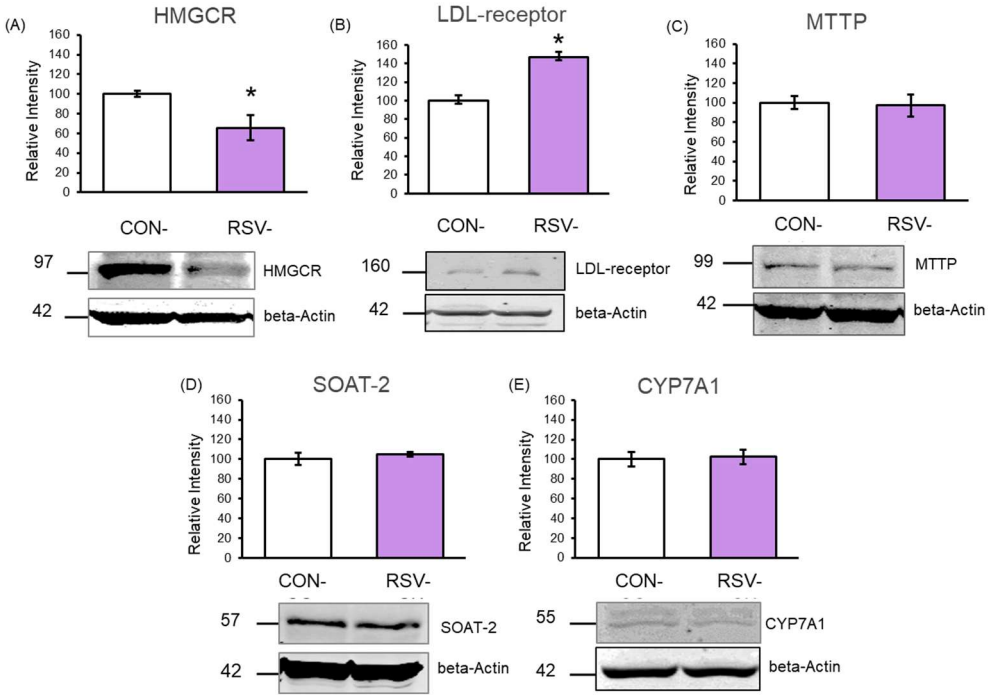

Figure 3. Protein expressions of HMGCR (A), LDLR (B), MTTP (C), SOAT-2 (D), and CYP7A1 (E) in the liver of male rat offspring at 36 weeks.

For each condition densitometric analysis was conducted relative to beta-actin. The left column indicates CON offspring, and the right column indicates RSV offspring. CON offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV offspring: a control diet during gestation, 20 mg/body weight RSV added once a day in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared to CON offspring.

The protein levels of SREBP-2, Insig-1, S1P and S2P

SREBP-2, a crucial transcription factor involved in cholesterol metabolism was examined. In this study, although higher precursor-SREBP-2 and Insig-1 levels were observed in RSV offspring (Figure 4(A), Figure 4(C) and Figure 4(F)), there was no significant difference in the levels of mature-SREBP-2 and the two proteases, S1P and S2P, between the two dietary groups (Figure 4(B), Figure 4(D) and Figure 4(E)).

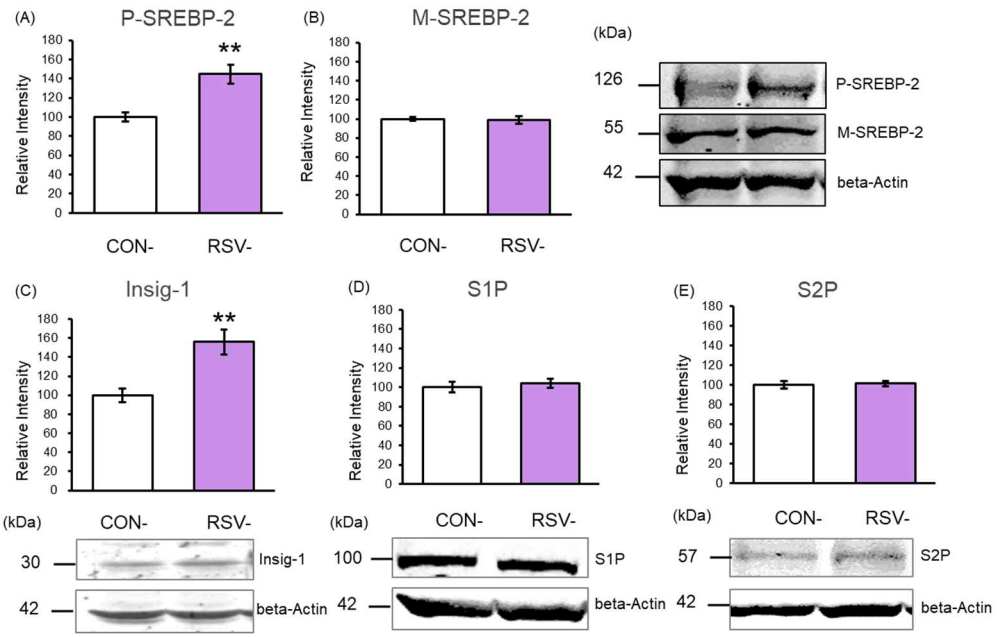

Figure 4. Protein expressions of precursor-SREBP-2 (A), mature-SREBP-2 (B), Insig-1 (C), S1P (D), and S2P (E) in the liver of male rat offspring at 36 weeks.

For each condition densitometric analysis was conducted relative to beta-actin. The left column indicates CON offspring, and the right column indicates RSV offspring. CON offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV offspring: a control diet during gestation, 20 mg/body weight RSV added once a day in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean ± SEM (n = 6). ** $p < 0.01$ compared to CON offspring.

Discussion

In this study, it is showed that maternal RSV intake during lactation reduces plasma cholesterol in male rat offspring at 36 weeks of age. Previous studies have indicated that HMGCR and LDL-receptor play crucial roles in cholesterol biosynthesis and lipid uptake respectively (Brown & Goldstein 1983; Geelen et al. 1986; Goldstein & Brown 1990; Spady 1992; De Faria et al. 1996). This suggested that in this study, suppression of hepatic cholesterol biosynthesis and promotion of cholesterol uptake into the liver from the blood contributed to the change in cholesterol localization, instead of altered cholesterol supply into blood or conversion into bile acid. To the best of our knowledge, this is the first report presenting the long-term effects of maternal RSV intake during lactation on cholesterol metabolism in male rat offspring and the first study to investigate the mechanisms underlying these effects. The effects and mechanism observed in RSV offspring to lower plasma cholesterol resemble the mechanism mediated by statins. Namely, it was similar in terms of reduction in blood cholesterol level via suppression of hepatic biosynthesis and promotion of cholesterol uptake from the blood.

Since expressions of hepatic HMGCR and LDL-receptor are regulated by SREBP-2 (Horton et al. 1998; Sakakura et al. 2001), in this study, the expression and activation of SREBP-2 were examined to elucidate the mechanism responsible for

changes in HMGCR and LDLR levels. This study showed a significant higher level of hepatic precursor-SREBP-2 in RSV offspring, but no significant difference was observed for hepatic mature-SREBP-2 level between the two groups. It has previously been reported that SREBP-2 expression is promoted during intracellular cholesterol depletion to increase cholesterol level by the expression of its target protein (Sato et al. 1996; Yang et al. 2002). Therefore, we hypothesized that although SREBP-2 expression in RSV offspring was promoted to increase hepatic cholesterol content, SREBP-2 was not activated and remained in its precursor, inactive state for some reason. We observed a result supporting this hypothesis, a higher level of hepatic Insig-1 in RSV offspring. Insig-1 is known to anchor precursor-SREBP-2 to ER and inhibit its maturation (Sun et al. 2005). Therefore, it is suggested that hepatic SREBP-2 maturation in RSV offspring was inhibited by upregulation of Insig-1. On the other hands, previous studies have revealed that many mechanisms are involved in the activity of SREBP-2 and in the regulation of expression of its target protein (Sanchez et al. 1995; Sato et al. 1996; Hou et al. 2008; Walker et al. 2010; Liu et al. 2015), and RSV treatment was speculated to influence these mechanisms (Li et al. 2011; De Amicis et al. 2011). Our previous study showed that maternal RSV intake during lactation activated hepatic AMPK and upregulated Sirt1 protein in adult rat offspring (Tanaka et al. 2017). Li et al. (2011) reported that AMPK

inhibits SREBP-2 maturation and represses its target gene expression. And Walker et al. (2010) showed that Sirt1 represses SREBP-1 and SREBP-2 target gene expression. These observations suggest that AMPK and Sirt1 may contribute to the inhibition of SREBP-2 maturation and changes in levels of its target proteins observed in this study. Although no other detailed mechanism has been examined in this study, the results in this study suggested that inhibition of SREBP-2 activation was involved in the alteration of cholesterol metabolism in RSV offspring. Our previous study indicated that maternal RSV intake during lactation in a normal nutrition environment lowered plasma Tg level of adult rat offspring (Tanaka et al. 2017). In this study, no significant change was found in hepatic Tg level in adult offspring due to maternal RSV intake during lactation. Therefore, maternal RSV intake showed a common effect on the metabolism of both lipids, lower lipid levels in plasma while maintaining hepatic lipids levels. Our previous study also showed lower levels of FA synthetases, FAS and ACC, and inhibition of SREBP-1 maturation which regulates expressions of FA synthases (Tanaka et al. 2017). And the present study showed a lower level of HMGCR, which is rate-determining protein of cholesterol synthesis, and a higher level of LDL-receptor, which can uptake both Tg and cholesterol, and inhibition of SREBP-2 maturation. This suggests that maternal RSV intake during lactation lower plasma lipids through attenuation of

lipogenesis and promotion of lipid uptake. Moreover, it is inferred that the inhibition of SREBPs maturation is important in the mechanism.

Several researchers have studied the effects of maternal RSV intake on offspring (Vega et al. 2016; Ros et al. 2018; Sun et al. 2019). Among the researches, the experimental design employed by Vega et al. (2016) was similar to this study in terms of animal species used, dose of RSV, and offspring age. However, the results obtained in terms of cholesterol metabolism are different. Vega et al. (2016) did not observe significant difference in serum cholesterol level of offspring between different groups. This suggested that the duration of exposure to RSV is important in gauging its impacts on cholesterol metabolism in rat offspring. In this study, mother rats were ingested RSV during lactation, but in research by Vega et al. (2016), mother rats were ingested RSV during gestation. This hypothesis was supported by previous studies that suggested that lactation period is more effective for the regulation of lipid metabolism in the offspring (Gregorio et al. 2010; Sun et al. 2012; Sarker et al. 2019).

Previous studies have reported that direct RSV treatment decreased the levels of plasma cholesterol and hepatic HMGCR in in vivo, and increased LDL-receptor in in vitro studies (Cho et al. 2008; Zhu et al. 2008; Do et al. 2008; Yashiro et al. 2012). Interestingly, similar effects in adult rat offspring at 36 weeks of age were observed even

though only the mothers were fed with RSV during lactation, for 3 weeks. Previous studies have reported that maternal nutrition causes long-lasting effects on health, including cholesterol metabolism, in offspring (Samuelsson et al. 2008; Sun et al. 2012; Ribaroff et al. 2017). In addition, previous studies have shown that dietary polyphenols transfer to offspring through breast milk (Franke et al. 2006; Song et al. 2013; Romaszko et al. 2014; Khymenets et al. 2016). Therefore, it is considered that the effects of exposure to RSV via breast milk last through adulthood. To the best of our knowledge, this is the first study showing the long-term effects of maternal RSV supplementation during lactation on cholesterol metabolism in rat offspring. Although the mechanism behind such effects was not revealed in this study, previous studies have indicated that these long-lasting effects could be caused by epigenetic mechanisms such as DNA and histone modifications. Several studies have reported that the activation of PPARα in mother mice during lactation leads to long-term downregulation of lipogenesis in offspring through attenuation of DNA methylation (Ehara et al. 2015; Yuan et al. 2018). Other studies have indicated that maternal polyphenol intake could modify the expression of epigenetic modulators in offspring (Sun et al. 2013; Tanaka et al. 2017; Kataoka et al. 2018). Furthermore, Cong et al. (2012) indicated that maternal low-protein diet during pregnancy and lactation altered cholesterol metabolism via changes in DNA methylation

and histone modification status in the promoter regions of cholesterol metabolism related proteins in the offspring. These studies suggested that maternal RSV ingestion during lactation influences epigenetic mechanism in offspring. Thus, further investigations are needed since epigenetic alterations could be crucially involved in the changes observed during this study.

Conclusion

In this study, it is showed that maternal RSV intake during lactation attenuates plasma cholesterol level while maintaining hepatic cholesterol content in adult male rat offspring. And it is suggested that maternal RSV treatment suppress hepatic cholesterol biosynthesis and promote hepatic cholesterol uptake from the blood. Additionally, these results indicated that maternal RSV intake during lactation elicits long-lasting effects on lipid metabolism in the rat offspring.

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CHAPTER 2

The protective effects of maternal green tea extract intake during lactation against hepatic lipid accumulation in adult male rats exposed to a continuous high-fat diet from the foetal period

Abstract

Background: Excess maternal lipid consumption during gestation and lactation can induce non-alcoholic fatty liver disease (NAFLD) in children. Green tea extract (GTE) contains abundant polyphenol, and it has been reported that it improves lipid metabolism and prevents NAFLD.

Objective: The aim of this study was to examine the effects of maternal green tea extract intake during lactation on hepatic lipid accumulation in adult male rats exposed to a continuous high-fat (HF) diet from the foetal period.

Materials and Methods: Pregnant Wistar rats received diets containing 13% (control-fat, CON diet) or 45% (HF diet) fat. CON-fed mothers received the same diet during lactation, whereas HF diet-fed mothers received either HF diet alone, or HF diet supplemented with 0.24% GTE. At weaning, male offspring were divided into three groups: CON/CON/CON, HF/HF/HF (HF-offspring), or HF/HF+GTE/HF (GTE-offspring) and were fed until 51 weeks.

Results: A significant hepatic triglyceride (TG) and cholesterol accumulation was observed in the HF-offspring as compared to the other offspring. This is presumed to be caused by the promotion of TG synthesis derived from exogenous fatty acid due to a significant higher level of diacylglycerol O-acyltransferase 1, and an attenuated TG

expenditure caused by lower levels of microsomal triglyceride transfer protein (MTTP) and long-chain acyl-CoA dehydrogenase. On the other hand, attenuated hepatic TG and cholesterol accumulation was observed in the GTE-offspring. The levels of the hepatic lipid metabolism-related enzymes were improved to the same level as the CON-offspring, and particularly, MTTP was significantly higher as compared with the HF-offspring.

Conclusion: This study indicated the potential protective effects of maternal GTE intake during lactation on HF diet-induced hepatic lipid accumulation via amelioration of hepatic lipid metabolism disorder in adult male rat offspring. It is suggested that maternal GTE intake suppressed lipid endocytosis and promoted lipid supply into the blood as the underlying mechanism.

Key words: maternal supplements; high-fat diet; green tea extract; adult offspring; hepatic fat accumulation

Introduction

In current society, non-alcoholic fatty liver disease (NAFLD) is one of the major health issues worldwide. Simple fat accumulation in the liver is called steatosis, and steatosis with inflammation is called steatohepatitis. NAFLD exists on a spectrum from simple fatty liver to an inflamed fatty liver (non-alcoholic steatohepatitis; NASH) (Ludwig et al. 1980; Schaffner & Thaler 1986; Younossi et al. 1998). The global prevalence of NAFLD in the general population has been estimated to be 25% whereas the global prevalence of NASH has been estimated to range from 3% to 5% (Vernon et al. 2011; Younossi et al. 2016; Younossi et al. 2018). NAFLD, especially NASH can progress to more serious disease stages, such as advanced fibrosis, cirrhosis, liver failure, or liver cancer (Torres et al. 2012). Global age-standardized prevalence in 2017 was estimated 11.061 cases per 100 000 for cirrhosis due to NASH and 1.20 cases per 100 000 for liver cancer due to NASH (James et al. 2018).

Many studies are ongoing to treat or prevent NAFLD, and plant extracts have been used as treatments in many studies. Green tea is a popular beverage made from the dried leaves of Camellia sinensis. Green tea extract (GTE) contains abundant polyphenolic compounds including catechin, epicatechin, gallocatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG). Among these

components, EGCG is the most abundant tea polyphenol (Graham 1992). GTE and EGCG have been well studied owing to their various beneficial effects on human diseases, including obesity, diabetes, liver diseases, inflammatory diseases, and cancer (Kao et al. 2006; Chu et al. 2017; Chen et al. 2018; Mahmoodi et al. 2020; Musial et al. 2020). GTE and its components have various therapeutic effects on lipid metabolism in animals fed a high-fat (HF) diet. Previous studies have reported that GTE and EGCG downregulate the expression of hepatic fatty acid synthases such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), sterol regulatory element-binding protein 1 (SREBP-1), and triglyceride (TG) synthase such as diacylglycerol O-acyltransferase 1 (DGAT-1) and diacylglycerol O-acyltransferase 2 (DGAT-2) (Santamarina, Oliveira, et al. 2015; Li et al. 2016; Bae et al. 2018). GTE has also been reported to have the effect of activating beta-oxidation in mitochondria and suppressing fatty acid uptake (Santamarina, Carvalho-Silva, et al. 2015; Huang et al. 2018).

It is widely known that personal lifestyle plays an essential role in hepatic lipid accumulation. On the other hand, studies have indicated that the environment in early life is an important factor of the cause and development of the NCDs including NAFLD. Among many environmental factors, maternal diet can be crucial. Epidemiological studies show that maternal malnutrition induces the risk of hyperglycemia and type 2

diabetes in adulthood (Ravelli et al. 1998; Li et al. 2010). And animal studies reported that maternal high-fat (HF) diet intake can affect the risk of NAFLD developing in offspring (Bruce et al. 2009; Gregorio et al. 2010; Ribaroff et al. 2017; Li et al. 2017; Sheen et al. 2018).

Maternal GTE intake is expected to have protective effects against diet-induced diseases in children as shown in researches (Sato et al. 2013; Hachul et al. 2018; Kataoka et al. 2018). Moreover, maternal polyphenol intake may be effective treatment to prevent diet-induced diseases in children. Li et al. (2012) reported that maternal GTE intake added to HF diet improved metabolic disorders in rat offspring more than GTE intake by the offspring itself after weaning. However, several studies pointed a concern about the adverse effects of maternal GTE intake during pregnancy (Alemdaroglu et al. 2007; Okubo et al. 2015; Otake et al. 2018). Our previous studies indicated that maternal polyphenol intake during lactation attenuated hepatic lipogenesis in adult rat offspring (Tanaka et al. 2017; Yamasaki et al. 2020). And other studies inferred that maternal HF diet intake during lactation strongly induces obesity in offspring than during pregnancy (Gregorio et al. 2010; Sun et al. 2012; Sarker et al. 2019). Additionally, maternal GTE intake during lactation showed no adverse effect on mothers and offspring in previous studies (Sato et al. 2013; Kataoka et al. 2018).Studies have demonstrated long-term

protective effects of maternal GTE intake against diet-induced kidney diseases in rat offspring (Sato et al. 2013; Kataoka et al. 2018). However, there are limited studies about the long-term effects of maternal GTE intake during lactation on potential hepatic lipid accumulation of HF diet-fed offspring.

Objective

This study was conducted to examine the effects of maternal GTE intake during lactation on hepatic lipid accumulation in adult male rat offspring exposed to maternal and post-weaning HF diet.

Materials and Methods

Animal treatment

The Animal Research Committee, Aomori University of Health and Welfare, approved this study, and all experimental procedures were performed following the Institutional Guidelines for Animal Experimentation. Seven-week-old virgin female Wistar rats obtained from CLEA Japan, Inc., (Tokyo, Japan) were maintained at a constant temperature of 23 ℃ ± 1 ℃ under a 12:12 hour light/dark cycle with ad libitum access to a commercial laboratory diet (MF diet; Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water. At 12-13 weeks of age, we determined whether the female rats were in the appropriate oestrus cycle stage for mating using a vaginal impedance reader (Model MK-10C; Muromachi Kikai Co. Ltd., Osaka, Japan) routinely in the afternoon. A reading >3 kΩ indicated that the female rats were in proestrus and presumably in oestrus. One appropriate female was mated with one male overnight. The presence of a vaginal plug the next morning indicated successful mating, and this day was noted as gestation day 0. As shown in Fig. 1, pregnant rats were randomly allocated to groups and fed control-fat diets (MF diet) (CON; n=5) or high-fat diets (HF; n=11) during gestation. The caloric content of the CON diet was 26% protein, 62% carbohydrate and 13% fat according to the manufacturer's information. The caloric content of the HF diet was 16% protein, 39%

carbohydrate and 45% fat. Following delivery, dams received either CON diet (CON/CON; n=5) or HF diet (n=11) during lactation. Those receiving the HF diet were further subdivided into receiving HF diet alone (HF/HF; n=6) or HF diet containing GTE 2.4g/kg diet (HF/HF+GTE; n=5). The GTE, Polyphenon E obtained from Mitsui-Norin Co. Ltd. (Shizuoka, Japan), contained 80%–98% total catechins by weight (the main component was EGCG, comprising ~65% of the material, as well as 0.4% caffeine). In this study, we administered 2.4g/kg diet of GTE, which showed no adverse effect on mothers and offspring on lactation in previous study (Sato et al. 2013; Kataoka et al. 2018). At weaning (22 days of age), six male offspring from each group of dams were randomly selected and divided into the following three groups based on provided diet: CON/CON/CON (CON-offspring; n=6), HF/HF/HF (HF-offspring; n=6), and HF/HF+GTE/HF (GTE-offspring; n=6). At 51 weeks of age, the male offspring were weighed, and blood samples were collected under anaesthesia after 12 hours fasting. The livers and adipose tissues were removed immediately, rapidly rinsed with ice-cold saline, and weighed. A portion of each liver was immediately frozen in liquid nitrogen and stored at -80 ℃ before evaluation.

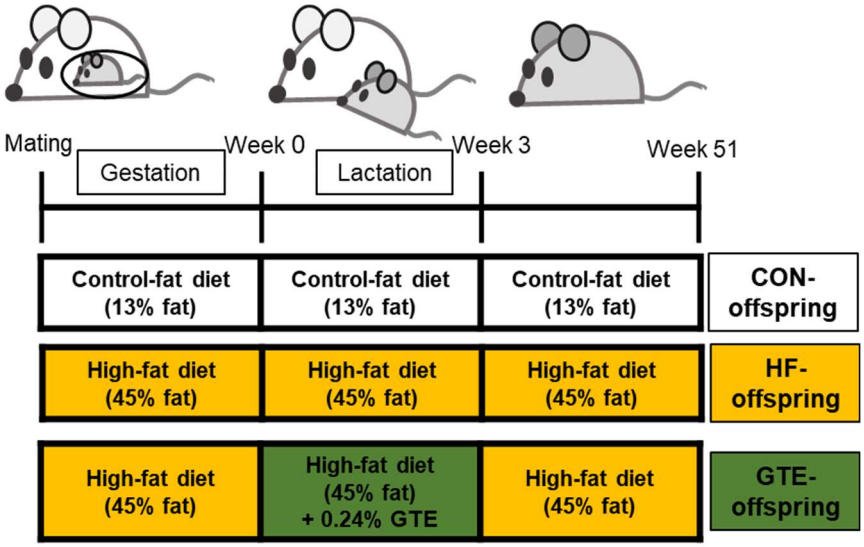

Figure 1. Experimental design.

CON-offspring: a control-fat diet (13% fat) during gestation and lactation, and after weaning, HF-offspring: a high-fat (HF) diet (45% fat) during gestation, lactation, and after weaning, GTE-offspring: HF diet during gestation and 0.24% GTE-containing HF diet during lactation, and HF diet after weaning.

Plasma lipids

Plasma samples were separated by centrifugation and tested for TG and total cholesterol levels using a commercially available kits (Wako Pure Chemical Industries Ltd., Osaka, Japan) according to the manufacturer's instructions.

Hepatic lipids

The liver sample was added to a solution of chloroform and ethanol (1:2, v/v) in an Eppendorf tube and homogenized. After incubation, chloroform was added to the

mixture and blended. Next, distilled water was added and mixed again. The chloroform layer was separated from the samples after centrifugation. The lipid fraction was obtained by evaporating chloroform with an evaporator and was dissolved in isopropanol. Lipid levels were tested for TG and cholesterol using a commercially available kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) according to the manufacturer's instructions.

Western blot analysis

For western blot analysis, the liver samples were homogenised in a buffer on ice. The homogenate was centrifuged at 4 ℃, and the supernatant was collected. Next, the obtained supernatant was heated to avoid denaturation of protein and the protein concentration in the sample was measured by Bradford assay (Bradford 1976) using Protein Assay (BIO-RAD, Hercules, U.S.A.). Proteins in the sample were separated by SDS-PAGE and biotinylated protein molecular weight markers (M&S TechnoSystems, Inc., Osaka, Japan) were used as protein standards. Proteins were then electrophoretically transferred onto a nitrocellulose membrane using the iBlot transfer system (Thermo Fisher Scientific K.K., Tokyo, Japan). The nitrocellulose membrane was incubated overnight at 4 ℃ in a buffer containing 2% skim milk as a blocking solution. The

membrane was then washed and exposed to primary antibodies: ACC (3676; Cell Signaling TECHNOLOGY, Inc., Massachusetts, U.S.A.), cholesterol 7 alpha-hydroxylase (CYP7A1; bs-2399R; Bioss, Massachusetts, U.S.A.), DGAT-1 (GTX48577; Gene Tex, Inc., California, U.S.A.), DGAT-2 (NBP1-71701SS; Novus Biologicals, LLC, Colorado, USA), FAS (ab22759; Abcam, Tokyo, Japan), HMGCR (ab180615, Abcam, Tokyo, Japan), long-chain acyl-CoA dehydrogenase (LCAD; ab196655; Abcam, Tokyo, Japan), LDL-receptor (3839-30T; BioVision, California, U.S.A.), microsomal triglyceride transfer protein (MTTP; ab186446; Abcam, Tokyo, Japan), sterol O-acyltransferase-2 (SOAT-2; bs5020R; Bioss, Massachusetts, U.S.A.), SREBP-1 (sc-13551; Santa Cruz Biotechnology, Inc., Texas, U.S.A.), and beta-Actin (M177-3; MEDICAL & BIOLOGICAL LABORATORIES CO., LTD., Aichi, Japan), in the presence of a 1% blocking solution. Next, the membrane was again washed and exposed to secondary antibodies: anti-rabbit IgG IRDye 680 (926-68071; M&S TechnoSystems, Inc., Osaka, Japan) or anti-mouse IgG IRDye 800 (926-32210; M&S TechnoSystems, Inc., Osaka, Japan). Protein bands were quantified using Odyssey infrared imaging system (M&S TechnoSystems, Inc., Osaka, Japan) and protein levels were normalised against those of beta-Actin from the same sample.

Hepatic 8-hydroxy-2'-deoxyguanosine

To measure 8-hydroxy-2'-deoxyguanosine (8-OHdG), DNA was isolated from the livers using High Pure PCR Template Preparation Kit (11796828001; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Extracted DNA was hydrolyzed using 8-OHdG Assay Preparation Reagent Set (292-67801; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) according to the manufacturer's instructions. Thereafter, the 8-OHdG concentration was determined by a competitive ELISA (KOG-HS10/E; JaICA, Nikken Seil Co., Ltd., Japan) according to the manufacturer's instructions.

Histopathological analysis

For histological analysis, paraformaldehyde-fixed liver was embedded in paraffin and sections (4μm) were stained with hematoxylin and eosin (H&E). Inflammatory cell infiltration was evaluated based on the New Inuyama classification (Ichida et al. 1996). Cholestasis was evaluated qualitatively.

Statistical analysis

Statistical analyses were performed using BellCurve for Excel (Social Survey

Research Information Co., Ltd., Tokyo, Japan). Data was tested using one-way analysis of variance (ANOVA) followed by Fisher’s LSD test or tested using Kruskal-Wallis test followed by Steel-Dwass's multiple comparison test. Each value was expressed as mean ± SEM. In all cases, statistical significance was set at $p<0.05$.

Results

Weight gain between week 3 to week 50

Fig. 2 shows the weight gain between week 3 to week 50 of the three offspring groups. No significant difference was found between HF-offspring and CON-offspring. GTE-offspring had higher body weights than CON-offspring after week 22, and HF-offspring after week 14.

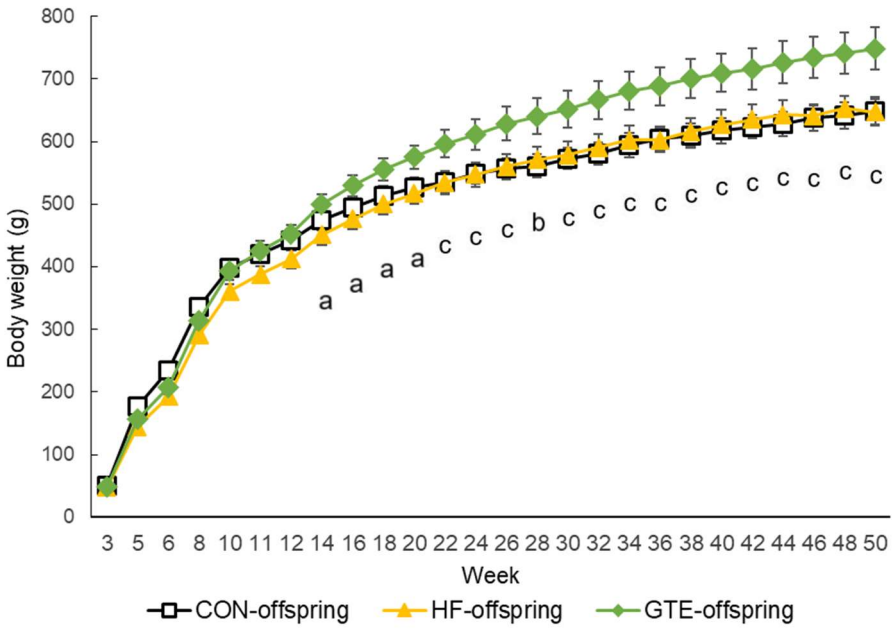

Figure 2. Weight gain of male rat offspring from week 3 to week 50.

The open square (□) indicates CON-offspring, the triangle (▲) indicates HF-offspring, and the closed square (◆) indicates GTE-offspring. CON-offspring: a control-fat diet during gestation and lactation, and after weaning, HF-offspring: a high-fat (HF) diet during gestation, lactation, and after weaning, GTE-offspring: HF diet during gestation and 0.24% GTE-containing HF diet during lactation, and HF diet after weaning. Values are expressed as mean ± SEM (n = 6). Data was tested using one-way ANOVA followed by Fisher's LSD test. a; $p<0.05$ GTE-offspring vs HF-offspring, b; $p<0.05$ GTE-offspring vs CON-offspring, c; $p<0.05$ GTE-offspring vs both HF-offspring and CON-offspring.

Morphological parameters at week 51

Table 1 shows morphological parameters at week 51. Significantly higher body weights were observed in GTE-offspring than in either of the other groups, while no significant difference was found between CON-offspring and HF-offspring. The liver of GTE-offspring was significantly heavier than HF-offspring. The kidney of HF-offspring was significantly lighter than the others. Epididymal fat of GTE-offspring was significantly heavier than CON-offspring. The perirenal fat in GTE-offspring was heavier than that of both CON- and HF-offspring. The relative liver and kidney weight of both HF-offspring and GTE-offspring was significantly lighter compared with it of CON-offspring. The relative epidydimal fat weight of only GTE-offspring was significantly heavier than CON-offspring. While the relative perirenal fat weight of GTE-offspring was much heavier than HF-offspring, both of them were significantly heavier than CON-offspring.

Table 1. Morphological parameters of male rat offspring at week 51.

Group	CON-offspring	HF-offspring	GTE-offspring
Body weight (week 3; g)	50.33±2.72[a]	47.33±2.85[a]	48.77±3.07[a]
Body weight[†] (week 51; g)	628.58±23.03[a]	621.13±11.0[a]	743.0±29.64[b]
Liver (g)	15.08±0.45[ab]	13.17±0.38[a]	16.05±0.97[b]
Kidney (g)	3.04±0.06[b]	2.58±0.07[a]	3.00±0.11[b]
Epididymal fat (g)	9.20±1.15[a]	12.04±0.82[ab]	15.51±2.19[b]
Perirenal fat (g)	24.79±2.30[a]	37.86±1.43[a]	60.39±5.85[b]
Liver/BW (g/kg)	24.09±0.84[b]	21.19±0.38[a]	21.54±0.58[a]
Kidney/BW (g/kg)	4.87±0.18[b]	4.15±0.08[a]	4.05±0.06[a]
Epididymal fat/BW (g/kg)	14.54±1.53[a]	19.37±1.21[ab]	22.58±1.62[b]
Perirenal fat/BW (g/kg)	39.23±2.95[a]	60.99±2.20[b]	80.44±5.81[c]

CON-offspring: a control-fat diet during gestation and lactation, and after weaning, HF-offspring: a high-fat (HF) diet during gestation, lactation, and after weaning, GTE-offspring: HF diet during gestation and 0.24% GTE-containing HF diet during lactation, and HF diet after weaning. Values are expressed as mean ± SEM (n = 6). Data was tested using one-way ANOVA followed by Fisher's LSD test. Values with the different superscript letters in the same line are statistically different ($p<0.05$). † At sacrifice.

Triglyceride and total cholesterol concentrations in the liver and plasma

Table 2 shows lipid concentrations in the liver and plasma of offspring at 51 weeks of age. Significantly higher levels of hepatic TG and cholesterol, and significantly lower levels of plasma TG and cholesterol were observed in HF-offspring than in CON-offspring, while no significant differences were found between CON-offspring and GTE-offspring. A similar pattern of change was observed in hepatic and plasma cholesterol levels, with a significant difference between CON-offspring and HF-offspring found in both sources.

Table 2. Lipids in liver and plasma

Group	CON-offspring	HF-offspring	GTE-offspring
Liver Tg (mg/g wet tissue)	15.93 ± 2.66^{a}	29.63 ± 6.22^{b}	23.99 ± 2.73^{ab}
Liver cholesterol (mg/g wet tissue)	2.15 ± 0.17^{a}	3.00 ± 0.37^{b}	2.37 ± 0.14^{ab}
Plasma Tg (mg/dl)	150.47 ± 9.36^{b}	87.8 ± 10.38^{a}	166.47 ± 23.73^{b}
Plasma cholesterol (mg/dl)	81.71 ± 4.83^{b}	53.31 ± 1.93^{a}	69.79 ± 5.36^{ab}

Hepatic triglyceride (Tg) and total cholesterol (TC) content (mg/g wet tissue), and plasma Tg and TC level (mg/dL) in male rat offspring at week 51. CON-offspring: a control-fat diet during gestation, lactation and after weaning; HF-offspring: a high-fat (HF) diet during gestation, lactation and after weaning; GTE-offspring: HF diet during gestation, 0.24% GTE-containing HF diet during lactation and HF diet after weaning. Values are expressed as mean ± SEM (n = 6). Data were tested using one-way ANOVA followed by Fisher's LSD test. Values with the different superscript letters in the same line are statistically different ($P < 0.05$).

Changes in hepatic lipid metabolic enzymes and transcription factor

Proteins for lipogenesis

Significantly lower levels of hepatic ACC (Fig. 3A) and FAS (Fig. 3B) were observed in HF-offspring than in CON-offspring. In GTE-offspring, levels of those enzymes were lowered but not significantly changed. To examine why these enzymes were reduced, level of hepatic SREBP-1 was measured. Significantly lower levels of SREBP-1 were observed in HF-offspring than in CON-offspring; this attenuated level was recovered in GTE-offspring (Fig. 3C). The level of hepatic DGAT-1 was significantly higher in HF-offspring than in CON-offspring and in GTE-offspring, while no significant change was found between GTE-offspring and CON-offspring (Fig. 3D). Significantly lower DGAT-2 levels were observed in both HF-offspring and GTE-offspring than in CON-offspring (Fig. 3E). Hepatic HMGCR level was measured as a key protein for cholesterol biosynthesis. Fig. 3F shows that no significant change was found in HMGCR level between the three groups.

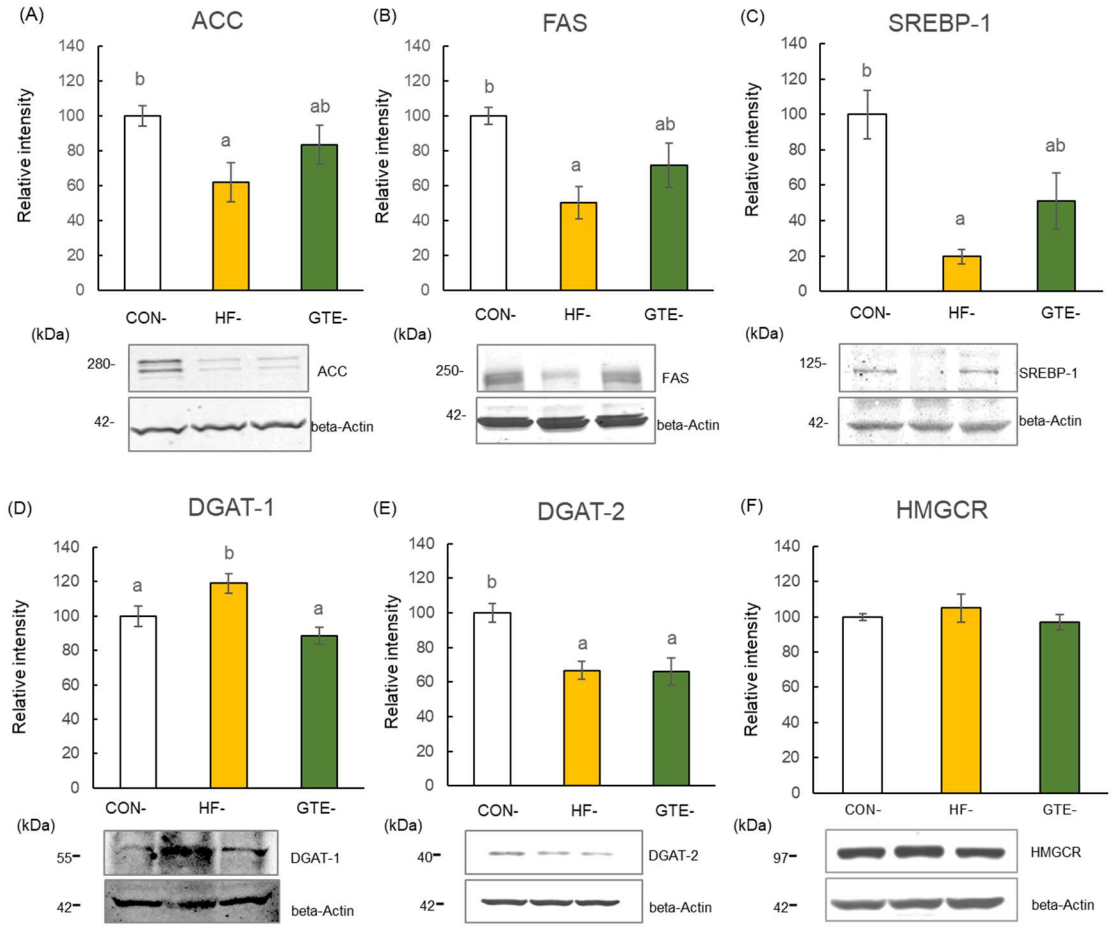

Figure 3. Protein expressions of acetyl-CoA carboxylase (ACC; A), fatty acid synthase (FAS; B), sterol regulatory element-binding protein 1 (SREBP-1; C), diacylglycerol O-acyltransferase 1 (DGAT-1; D), diacylglycerol O-acyltransferase 2 (DGAT-2; E), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR; F) in the liver of male rat offspring at week 51.

For each condition densitometric analysis was conducted relative to beta-actin. The left column indicates CON-offspring, the middle column indicates HF-offspring, and the right column indicates GTE-offspring. CON-offspring: a control-fat diet during gestation and lactation, and after weaning, HF-offspring: a high-fat (HF) diet during gestation, lactation, and after weaning, GTE-: HF diet during gestation and 0.24% GTE-containing HF diet during lactation, and HF diet after weaning. Values are expressed as mean ± SEM (n = 6). Data was tested using one-way ANOVA followed by Fisher's LSD test. The bars in the different serial shown by the different letter significantly differ from each other at a level of 5%.

Proteins for lipid supply into blood

Hepatic MTTP and SOAT-2 level were measured as crucial proteins to supply lipids into the blood. In HF-offspring, hepatic MTTP level was downregulated, but not significantly lower than CON-offspring ($p=0.0672$), while in GTE-offspring it significantly upregulated compared to HF-offspring, and no significant change was found between CON-offspring and GTE-offspring (Fig. 4A). No significant difference was found in hepatic SOAT-2 level between the three groups (Fig. 4B).

Protein for lipid uptake from the blood

Hepatic low-density lipoprotein (LDL)-receptor level was measured to evaluate hepatic lipid uptake from the blood. Fig. 4C shows that hepatic LDL-receptor level in HF-offspring was significantly higher than the other two groups.

Proteins for lipid expenditure; beta-oxidation and excretion

LCAD level was measured as an important role in mitochondrial fatty acid oxidation. A significantly lower hepatic LCAD level was observed in HF-offspring than in CON-offspring, while there was no significant change in GTE-offspring (Fig. 4D). To analyse cholesterol catabolizing pathways, hepatic CYP7A1 level was measured. In this study, no significant change was observed between the three groups (Fig. 4E).

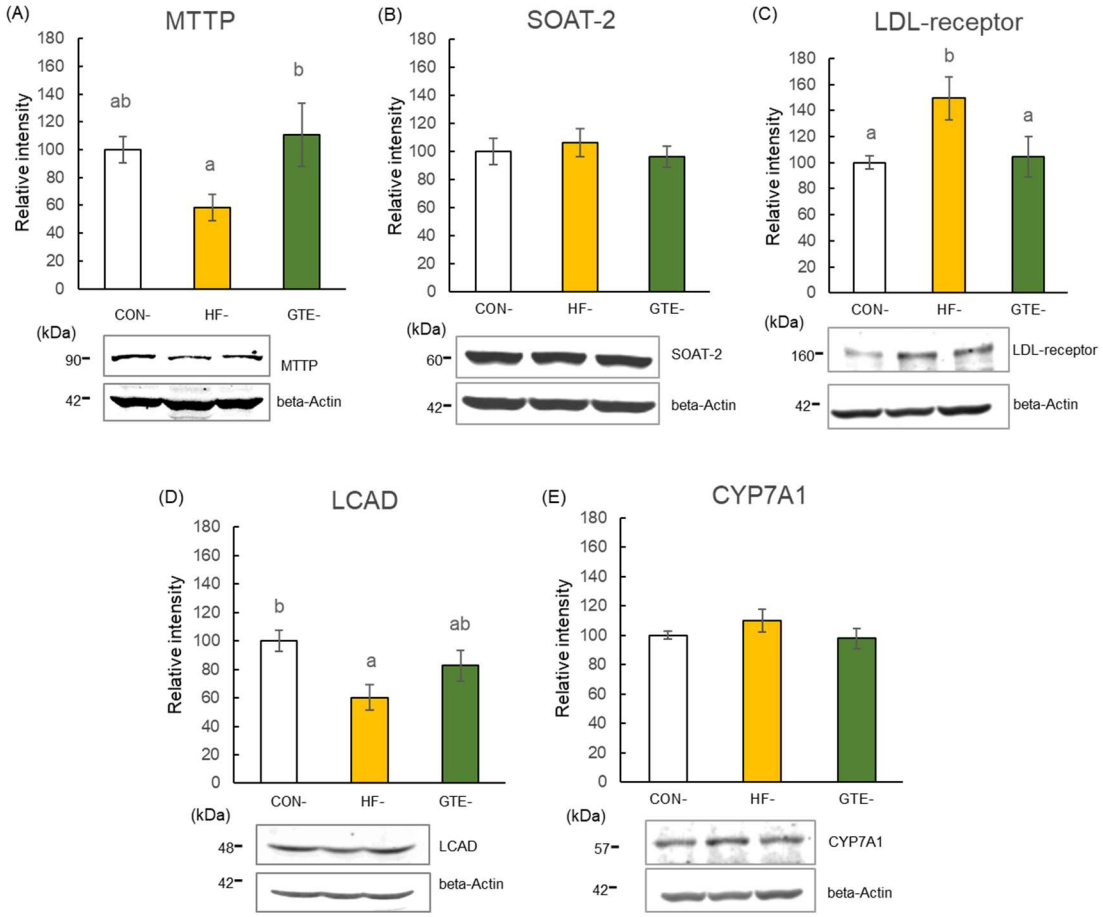

Figure 4. Protein expressions of microsomal triglyceride transfer protein (MTTP; A), sterol O-acyltransferase-2 (SOAT-2; B), LDL-receptor (C), long-chain acyl-CoA dehydrogenase (LCAD; D) and cholesterol 7 alpha-hydroxylase (CYP7A1; E) in the liver of male rat offspring at week 51.

For each condition densitometric analysis was conducted relative to beta-actin. The left column indicates CON-offspring, the middle column indicates HF-offspring, and the right column indicates GTE-offspring. CON-offspring: a control-fat diet during gestation and lactation, and after weaning, HF-offspring: a high-fat (HF) diet during gestation, lactation, and after weaning, GTE-offspring: HF diet during gestation and 0.24% GTE-containing HF diet during lactation, and HF diet after weaning. Values are expressed as mean ± SEM (n = 6). Data was tested using one-way ANOVA followed by Fisher's LSD test. The bars in the different serial shown by the different letter significantly differ from each other at a level of 5%.

Change in hepatic 8-OHdG concentration

Fig. 5 shows the hepatic 8-OHdG concentration in the liver of offspring at 51 weeks of age. Significantly higher 8-OHdG concentration was observed in HF-offspring compared to CON-offspring, while no significant difference was observed between HF-offspring and GTE-offspring or between GTE-offspring and CON-offspring.

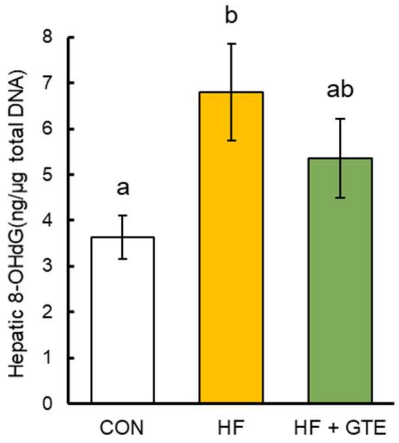

Figure 5. 8-OHdG in the liver of offspring at 51 weeks of age.

The left column indicates CON-offspring, the middle column indicates HF-offspring, and the right column indicates GTE-offspring. CON-offspring: a control-fat diet during gestation and lactation, and after weaning, HF-offspring: a high-fat (HF) diet during gestation, lactation, and after weaning, GTE-offspring: HF diet during gestation and 0.24% GTE-containing HF diet during lactation, and HF diet after weaning. Values are expressed as mean ± SEM (n = 6). Data was tested using one-way ANOVA followed by Fisher's LSD test. The bars in the different serial shown by the different letter significantly differ from each other at a level of 5%.

Histopathological analysis

Histological examination (Fig. 6 and Table 3) shows inflammatory cell infiltration and cholestasis in the liver of offspring. In the liver of HF-offspring, significant severe inflammatory cell infiltration was observed compared to the liver of CON-offspring. And cholestasis was observed in only the liver of HF-offspring.

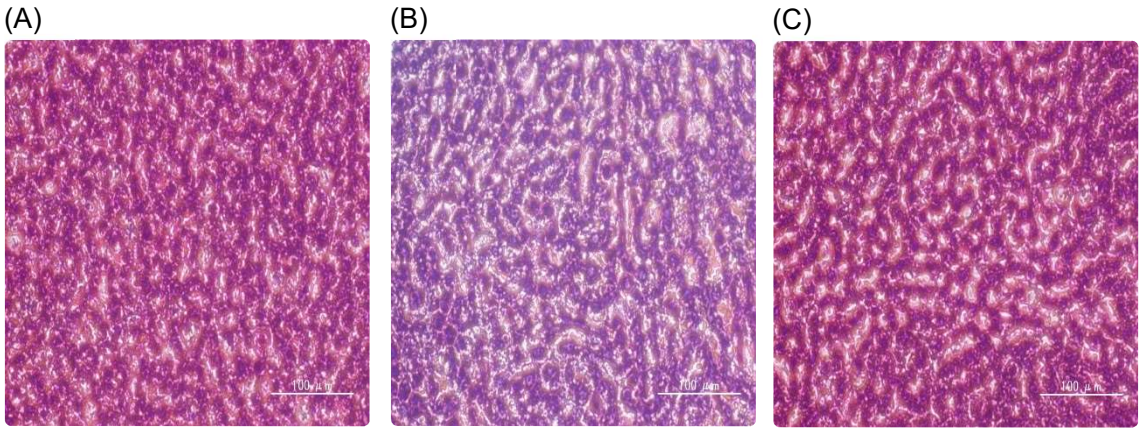

Figure 6. H&E stained liver of offspring at 51 weeks of age.

For histological analysis, paraformaldehyde-fixed liver was embedded in paraffin and sections (4μm) were stained with hematoxylin and eosin (H&E). A. CON-offspring: a control-fat diet during gestation and lactation, and after weaning, B. HF-offspring: a high-fat (HF) diet during gestation, lactation, and after weaning, C. GTE-offspring: HF diet during gestation and 0.24% GTE-containing HF diet during lactation, and HF diet after weaning.

Table 3. Histopathological analysis

Group	CON-offspring	HF-offspring	GTE-offspring
inflammatory cell infiltration			
A0	9	3	5
A1	0	5	3
A2	0	1	0
A3	0	0	0
statistical difference	a	b	ab
Cholestasis			
+	0	5	0
−	9	4	8
statistical difference	a	b	a

Inflammatory cell infiltration was evaluated based on the New Inuyama classification. Cholestasis was evaluated qualitatively. CON-offspring: a control-fat diet during gestation and lactation, and after weaning, HF-offspring: a high-fat (HF) diet during gestation, lactation, and after weaning, GTE-offspring: HF diet during gestation and 0.24% GTE-containing HF diet during lactation, and HF diet after weaning. Data was tested using Kruskal-Wallis test followed by Steel-Dwass's multiple comparison test. Values with the different letters in the same line are statistically different ($p<0.05$).

Discussion

This study examined hepatic lipid metabolism in adult male rat offspring, to investigate the effects of maternal GTE intake on hepatic lipid accumulation induced by maternal and post-weaning HF diet.

In HF-offspring livers, downregulated fatty acid synthesis, fatty acid oxidation, and de novo TG synthesis were observed, whereas exogenous TG synthesis was upregulated. There were also indications that lipid secretion from the liver was attenuated while lipid uptake was promoted.

ACC and FAS are widely known as crucial enzymes for de novo fatty acid synthesis from acetyl-CoA, and their expression is regulated by SREBP-1 (Shimomura et al. 1998). Our western blot results indicate lower expression of both ACC and FAS, and their transcriptional regulator SREBP-1, suggest that hepatic fatty acid synthesis in HF-offspring was attenuated through a transcriptional mechanism. Both DGAT-1 and DGAT-2 mediate the binding of diacylglycerol and acyl-CoA at the final stage of TG synthesis. However, each uses a different substrate, and it has been found that DGAT-1 uses exogenous substrates--fatty acid derived from diet--whilst DGAT-2 uses de novo fatty acid for its substrate (Wurie et al. 2012). LDL-receptor mediates the endocytosis of lipoproteins which contain apo-B100 and apo-E, including LDL, VLDL, and chylomicron remnant into the liver (Mahley 1988; De Faria et al. 1996; Williams 2008),

and it plays important role in lipid uptake both TG and cholesterol (Brown & Goldstein 1983; Spady 1992). In HF-offspring, it is inferred that increased exogenous fatty acid influx into the liver by uptake from the blood via LDL-receptor suppressed de novo fatty acid synthesis and promoted TG biosynthesis from exogenous fatty acid. Thus, changes in the expression of the two TG synthases can be related to changes in the amount of each substrate. Donnelly et al. (2005) reported that about 60% of the lipids contained in the liver were derived from exogenous fatty acids; this suggests that a significant higher DGAT-1 level in HF-offspring demonstrated in this study contributed to the hepatic lipid accumulation seen in HF-offspring. It has also been reported that a mechanism exists to prevent excessive accumulation of lipids even if TG synthesis is extraordinarily promoted. Yamazaki et al. (2005) reported that overexpression of DGAT-1 promoted TG synthesis, whereas hepatic lipid accumulation was not significant due to promoted TG secretion with VLDL. However, in this study, the level of hepatic MTTP, an enzyme that greatly contributes to TG and cholesterol supply into blood with lipoprotein (Raabe et al. 1999; Hussain et al. 2012), was lowered in HF-offspring. Lower plasma lipid in HF-offspring supports the idea that lipid secretion from HF-offspring was attenuated. Moreover, LCAD, a protein that contributes to mitochondrial fatty acid oxidation (Kurtz et al. 1998), was also significant lower in HF-offspring. These results suggest that the mechanism that

prevents excessive accumulation of TG in the liver is not functioning correctly in the HF-offspring. It is speculated that TG accumulated in the liver of HF-offspring due to a lipid metabolic disorder: excess promotion of TG synthesis using exogenous fatty acid and of uptake from the blood, and attenuated lipid expenditure by secretion or oxidation. The mechanism of cholesterol accumulation in HF-offspring liver had been considered to be similar to it of TG accumulation. In this study, levels of HMGCR, SOAT-2, MTTP, CYP7A1, and LDLR were measured to examine changes in cholesterol metabolism. HMGCR is the rate-determining enzyme of cholesterol synthesis (Geelen et al. 1986; Goldstein & Brown 1990). SOAT-2 and MTTP contribute to cholesterol supply into the blood. SOAT-2 esterifies free cholesterol in hepatic lipid droplets with acyl-CoA (Chang et al. 2009; Marshall et al. 2014), while MTTP associates cholesterol ester and TG with apo-B protein to form VLDL (Gordon & Jamil 2000; Hussain et al. 2012). CYP7A1 converts cholesterol into bile acid and plays important role in cholesterol excretion pathways (Chiang 2009). No significant change was found in levels of HMGCR, SOAT-2, and CYP7A1 compared to CON-offspring. Changes in levels of MTTP and LDLR, which contribute to both TG and cholesterol metabolism were observed as described in the former paragraph. It indicates that cholesterol accumulated in the HF-offspring liver due to uptake from the blood and attenuated supply into the blood, but not biosynthesis

or expenditure.

The changes in levels of hepatic lipid metabolism-related proteins in the GTE-offspring showed a similar pattern as that seen in the HF-offspring in most proteins, but unlike in the HF-offspring, the changes were not significant compared to the CON-offspring. This indicates that maternal GTE intake ameliorated abnormalities in hepatic lipid metabolism in the offspring. Only the level of DGAT-2 was significantly lower in GTE-offspring than the CON-offspring. It is supposed to be related to that de novo fatty acid synthesis tended to be attenuated from the CON-offspring and that fatty acid oxidation was performed normally. Level of MTTP in GTE-offspring was significantly higher than in HF-offspring. As HF diet intake reduces MTTP protein levels (Wang et al. 2014), this suggests that GTE intake during lactation modulates MTTP expression in the long term. Moreover, reports have shown an association between lipid secretion from the liver and hepatic lipid accumulation (Raabe et al. 1999; Yamazaki et al. 2005). And hepatic LDL-receptor level in GTE-offspring was significantly lower than in HF-offspring suggesting reduced lipid uptake into the liver. These observations suggest that maternal GTE intake suppressed HF diet-induced hepatic TG and cholesterol accumulation in offspring by attenuation of hepatic lipid metabolic disorder particularly lipid supply into the blood and lipid uptake from the blood.

Changes in liver lipid metabolism in the HF group suggest that the liver is injured. Yamazaki et al. (2005) suggests that even if lipid synthesis in the liver becomes excessive, the liver has a mechanism that maintains the homeostasis of its lipid concentration by increasing the amount supplied to the blood. On the other hand, Fujita et al. (2009) reported that MTTP expression was decreased in the liver of NASH patients, which is a more severe condition than simple fatty liver. This study shows that significantly increased DGAT-1 level and decreased MTTP level in HF-offspring suggesting hepatic lipid metabolism disorder and liver injury in HF-offspring. Increased 8-OHdG, which is DNA oxidative marker, significant inflammatory cell infiltration, and cholestasis observed in the liver of HF-offspring supports this idea. Besides, it has been suggested that promoted hepatic fat synthesis may inhibit adipose tissue development (Shimano et al. 1996). This may be related to that the HF group did not gain significant weight compared to the CON group despite being bred on a high-fat diet. In the GTE group liver, lipid metabolism in the liver--the function of regulating lipid concentration-- is maintained, which is considered to have led to attenuation of lipid accumulation in the liver, development of adipose tissue, and weight gain. However, further studies are needed to elucidate the underlying mechanisms and impacts on health.

One of the key findings in this study was that lipid metabolism in the HF- and

GTE-offspring were significantly different at 51 weeks of age. In addition, from the change in weight gain from week 14 to week 51, it can be inferred that the changes in metabolism have been continuously persisted. This indicates long-term protective effects of short-term treatment for the mother during lactation on hepatic lipid accumulation in children. Polyphenols is transferred to the child via breast milk, but EGCG, the main component of GTE, has been shown to be rapidly excreted and not retained (Chen et al. 1997; Lambert et al. 2003; Song et al. 2013; Khymenets et al. 2016). Therefore, it is considered that the GTE itself, taken by the mother during lactation, did not remain in the offspring but affected the early life of offspring, and these effects were maintained until late adulthood. Epigenetic alteration, which is heritable changes in gene function that take place without a change in the DNA sequence is one of the possible mechanisms that may play a role in maintaining these effects from childhood to late adulthood. Previous studies have shown that the expression of some lipid metabolism-related proteins is under epigenetic regulation (Ehara et al. 2012; Wang et al. 2014; Gracia et al. 2014), which can be induced during early life by maternal nutrient status. Ehara et al. (2012) reported that maternal HF diet intake reduces DNA methylation of glycerol-3-phosphate acyltransferase 1 promoter region in offspring and promotes TG synthesis. Other studies (Ehara et al. 2015; Yuan et al. 2018) also showed treatment for mothers modulates

epigenetic regulation of lipid metabolism-related gene expression in offspring. Moreover, it has also been suggested that epigenetic alterations that occur during early life can be preserved until post-growth (Reizel et al. 2015). From previous studies, it is also inferred that there are long-term effects of GTE intake on the expression of DNA methyltransferase 1 and 3a, which act in a compensatory manner against maternal malnutrition (Sun et al. 2013; Kataoka et al. 2018). The hepatic lipid accumulation observed in HF-offspring may have been caused by maternal HF diet-induced epigenetic alterations in hepatic lipid metabolism. It has been suggested that maternal GTE intake during lactation has effects of improving hepatic lipid metabolism in offspring through epigenetic alterations. On the other hand, there are many possible factors that may contribute to hepatic lipid accumulation. It has been reported that insulin resistance and chronic inflammation contribute to hepatic lipid accumulation (Kitade et al. 2017). There are also suggested effects of maternal HF diet and GTE intake on the formation of predispositions to hepatic lipid accumulation in offspring (Li et al. 2012; Hachul et al. 2018). Therefore, further study is necessary to clarify the mechanism underlying the preventive effects of maternal GTE intake on hepatic lipid accumulation.

Conclusions

In this study, it was examined that the effects of maternal GTE intake during lactation on hepatic lipid metabolism in adult male rat offspring exposed to a continuous high-fat diet from the foetal period to late adulthood. It was revealed that HF diet induces hepatic lipid accumulation into male rat offspring at 51 weeks of age. And maternal GTE intake during lactation showed protective effects against HF diet-induced hepatic lipid accumulation. In male rat offspring exposed to maternal and post-weaning HF diet, hepatic lipid accumulation was induced by hepatic lipid metabolism disorder; promoted TG synthesis and lipid uptake, and suppression of hepatic lipid expenditure. It was shown that GTE intake during lactation improved hepatic lipid metabolism, including lipid supply into the blood and lipid uptake from the blood, and suppressed hepatic lipid accumulation while induced adipose tissue development and body weight gain. It is suggested that maternal GTE intake during the lactation affects offspring until late adulthood through epigenetic alterations. This study implies that maternal GTE intake during lactation is effective in protecting children from hepatic lipid accumulation, while further studies are needed to clarify the mechanism.

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SUMMARY

This thesis was aimed to examine the effects of maternal polyphenol intake on lipid metabolism in adult rat offspring and alterations in levels or activation of hepatic lipid metabolism-related proteins as a possible underlying mechanism of the effects.

In chapter 1, I studied the effects of maternal RSV intake during lactation on adult rat offspring in a normal nutritional environment. The study showed that maternal RSV intake during lactation attenuates plasma lipid (TG and cholesterol) level in adult offspring, while maintains hepatic lipid levels. As its mechanism, it is suggested that maternal RSV intake suppresses hepatic lipogenesis and promotes lipid uptake from plasma into the liver. Our previous study examining the effects of maternal RSV intake on adult offspring reported lower blood triglycerides and suppression of fatty acid synthesis in the liver. This suggests that maternal RSV intake during lactation induces similar effects on TG and cholesterol metabolism in adult offspring. And it is implied that the effects of maternal RSV intake on lipid metabolism in offspring depend on when mothers take RSV. In other words, the lactation period may be the duration when the maternal RSV intake affect most effective for lipid metabolism in offspring.

In chapter 2, I studied the effects of maternal GTE intake on adult rat offspring in the excessive nutritional environment. The study showed that maternal GTE intake

during lactation attenuates hepatic lipid (TG and cholesterol) accumulation in adult rat offspring exposed to continuous HF diet from foetal period. As its mechanism, it is suggested that GTE treatment ameliorated HF diet-induced lipid metabolic disorder as the nearly same as the control group. Maternal GTE intake suppresses hepatic lipid uptake and promotes lipid supply from plasma into the liver. In TG metabolism, in addition, GTE treatment suppressed TG synthesis while recovered FA synthesis and FA oxidation. Interestingly, continuous HF diet exposure did not significantly increase the body weight of offspring, whereas HF diet exposure adding maternal GTE intake during lactation obviously increased the bodyweight of offspring. Observations in this study suggest that hepatic lipid metabolic disorders induced by continuous exposure to HF diet-induced hepatic lipid accumulation and inhibited the development of adipose tissue. Moreover, amelioration of the hepatic lipid metabolism disorder by maternal GTE treatment attenuated lipid accumulation in the liver and promoted the development of adipose tissue which leads to bodyweight gain.

My research revealed that maternal polyphenol intake during lactation is effective not only in a normal nutritional environment but also in the HF diet, excessive nutritional environment. It is suggested that maternal polyphenol intake during lactation alters hepatic lipid metabolism pathways in particular biosynthesis, uptake from the blood,

and lipogenesis. It is speculated that maternal polyphenol intake during lactation induces epigenetic alterations in offspring, while further investigation is needed to clarify the mechanisms of alterations in protein expression in the liver of offspring observed in this study. Since this study focused on alterations in adulthood of offspring, it is a future issue to examine sequential alterations by maternal polyphenol intake that occur along with the development of offspring. Although further investigations are needed, this study showed the potential protective effects of maternal polyphenol intake during lactation against future diseases such as lipid metabolic disorders in offspring.

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