Effect of anti-oxidants, Ricetrienol and α-tocopherol, on adipocytokine abnormalities and fatty liver in Otsuka Long-Evans Tokushima Fatty diabetic rats

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ABSTRACT

Introduction: We investigated the effect of Ricetrienol, which is an anti-oxidant extracted from rice bran, and α-tocopherol on the adipocytokine abnormalities and fatty liver in Otsuka Long-Evans Tokushima Fatty (OLETF) rats.

Materials and Methods: A total of 18 OLETF rats were bred using a 30% sucrose solution (the diabetic group; DM), whereas another 18 OLETF rats were bred using ordinary water (the non-diabetic obese group; OB) as drinking water, respectively. After the sucrose-fed rats developed diabetes, all of the rats from the diabetic and obese groups were randomly divided into three groups. Then each group was fed either standard chow (DM-S, OB-S group), 0.05% Ricetrienol-containing chow (DM-R, OB-R group) or 0.05% α-tocopherol-containing chow (DM-A, OB-A group), respectively. After 12 weeks of feeding, all the rats were killed. Plasma insulin, adiponectin, resistin and leptin were assayed by enzyme immunoassay. Histopathological findings of liver tissue were scored according to Brunt and Kleiner's method, and triglyceride contents of the liver tissue were investigated.

Results: Plasma adiponectin was significantly reduced in DM-S compared with OB-S, but it had significantly increased in DM-R and DM-A as opposed to DM-S. Plasma resistin showed a significant increase in DM-S compared with OB-S, but it was significantly reduced in DM-A than in DM-S. Though the triglyceride contents of liver tissue significantly increased in DM-S as opposed to OB-S, they were significantly reduced in DM-R compared with DM-S. Histopathological scores were significantly higher in DM-S than OB-S.

Conclusions: The present study shows that Ricetrienol might prevent adipocytokine abnormalities and fatty liver in OLETF diabetic rats. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00090.x, 2011)

KEY WORDS: Adipocytokine, Anti-oxidant, Fatty liver

INTRODUCTION

Type 2 diabetes and metabolic syndrome are important risk factors of macrovascular[1–3] and liver disease[4,5]. It has been reported that adipocytokine abnormalities play a major role in the pathogenesis of macrovascular disease[6], and that oxidative stress might be one cause of these abnormalities[7]. Furthermore, recent studies have reported that a fatty liver can progress to end-stage liver disease in some patients with fatty liver disease[8] and that oxidative stress might be implicated as a key factor contributing to hepatic injury[5]. Therefore, prevention of excessive oxidative stress and adipocytokine abnormalities in patients with type 2 diabetes and metabolic syndrome is thought to be very important.

Several studies have reported that thiazolidinedione is effective in preventing adipocytokine abnormalities[9] and hepatic steatosis[10]. Telmisartan has also been reported to be effective in preventing the reduction of adiponectin[11,12]. Economically, however, it is unfeasible to give these medical drugs to all patients.

The efficacy of anti-oxidant therapy for the prevention of adipocytokine abnormalities and fatty liver has also been reported[13,14]. Although the clinical efficacy of anti-oxidant therapy has not been established in preventing the effects of atherosclerosis, administering supplements with an anti-oxidative effect to type 2 diabetics and patients with metabolic syndrome might reduce the occurrence of macrovascular disease. In order to use such supplements for atherosclerosis or fatty liver prevention, low cost and safety are vital.

Ricetrienol (RT) is an anti-oxidant that is extracted from rice bran, its contents are tocopherols (>3%), tocotorienol (>3%), squalene (>8%) and plant sterols (>10%). We have previously reported that RT had a protective effect against oxidative

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damage in an experiment with diabetic mice[15]. Therefore, RT might have a beneficial effect for the prevention of adipocytokine abnormalities and fatty liver. Furthermore, RT is approved as a food additive in Japan, and it is possible to produce it in large quantities at a low cost.

In the present study, we investigated the preventive effects of RT on adipocytokine abnormalities and fatty liver, and compared these with the effects of α-tocopherol (AT), a standard antioxidant, in Otsuka Long-Evans Tokushima Fatty (OLETF) rats.

MATERIALS AND METHODS

Experimental Design

A total of 36 male OLETF rats were provided by the Tokushima Research Institute of Otsuka Pharmaceutical Corporation (Tokushima, Japan). The OLETF rat is a useful animal model of spontaneous type 2 diabetes with obesity[16], and the onset age can be accelerated by using a high-sucrose diet[17].

Age at the start of the experiment was 5 weeks, with an approximate bodyweight of 110 g. All rats were maintained in a specific pathogen-free facility under a controlled temperature of 23°C with a humidity of 50% in a 12-h artificial light/dark cycle at Tsuno Food Industrial Co. Ltd. A total of 18 OLETF rats were bred using a 30% sucrose solution as drinking water as the experimental model of obese type 2 diabetes (DM group). The other 18 OLETF rats were bred using ordinary water as a model of non-diabetes with obesity (OB group). All rats were initially fed standard rodent chow for 12 weeks.

The diabetic state was confirmed by measurement of casual blood glucose exceeding 200 mg/dL. At age 17 weeks, all rats of the DM group satisfied the aforementioned criteria, but none of the OB group did. Rats in the DM and OB groups were randomly divided into three groups with matched bodyweight and blood glucose levels. Then, each group was fed standard chow (DM-S, OB-S group, $n = 6$), 0.05% RT-containing chow (DM-R, OB-R group, $n = 6$) or 0.05% AT-containing chow (DM-A, OB-A group, $n = 6$) for 12 weeks, respectively. Throughout the experiment, bodyweight was measured and blood glucose levels were monitored twice weekly by tail vein sampling using a self-monitoring glucose measuring system (Glucocard; Arkray, Kyoto, Japan). Persistent hyperglycemia was confirmed in the DM group.

At age 29 weeks, 24-h urine samples were collected using metabolic cages. On the last day of the experiment at age 29 weeks, after 8 h of fasting and blood glucose measurement,

Table 1 | Physical and laboratory data

	sBW (g)	eBW (g)	BG (mg/dL)	GHb (%)	Insulin (μU/mL)	HOMA-R	Adiponectin (ng/mL)	Resistin (ng/mL)	Leptin (pg/mL)	MCP-1 (ng/mL)	ICAM-1 (ng/mL)	8-Isoprostane (pg/mgCr)	TG-L (mg/g)
OB-S													
Mean	107	637	140	3.86	4.10	1.48	3.44	16.2	1695	6.97	44.6	47.3	59.2
SD	19	31	14	0.25	2.23	0.85	0.44	5.2	373	1.57	5.7	2.9	18.5
OB-R													
Mean	112	638	134	3.62	2.98	1.02	3.02	17.3	1542	5.11	36.1	56.8	64.9
SD	26	75	16	0.31	1.49	0.59	0.42	3.2	214	0.91	5.5	13.6	8.4
OB-A													
Mean	115	655	132	3.80	3.25	1.10	3.00	17.0	1763	5.98	37.0	55.7	66.8
SD	10	45	12	0.47	1.73	0.63	0.30	2.9	271	0.84	4.7	9.8	10.2
DM-S													
Mean	108	560	341[a]	8.32[a]	4.82	3.96[c]	2.16[a]	32.2[b]	1746	5.26	37.5	69.3[c]	121.4[a]
SD	10	127	85	1.15	3.27	2.72	0.46	9.6	813	3.41	7.7	17.8	20.7
DM-R													
Mean	120	531	306[a]	8.25[a]	3.81	2.08	3.64*	24.1	1797	4.58	38.1	59.7	96.2$
SD	26	71	106	1.50	2.95	1.31	0.62	12.3	695	0.69	4.0	28.3	22.9
DM-A													
Mean	112	573	323[a]	8.60[a]	4.01	3.18	2.98[#]	19.6$	1952	5.82	38.7	58.9	114.9
SD	13	97	107	1.02	2.32	2.00	0.52	11.0	576	3.02	6.0	18.3	15.5
P-value	NS	NS	<0.001	<0.001	NS	<0.05	<0.001	<0.05	NS	NS	NS	<0.05	<0.001

P-value indicates the statistical significance analized by ANOVA. BG, blood glucose; DM-A, obese type 2 diabetes group fed 0.05% α-tocopherol containing chow DM-R, obese type 2 diabetes group fed 0.05% Ricetrienol-containing chow; DM-S, obese type 2 diabetes group fed standard chow; eBW, body weights at the end of the experimental period; GHb, glycated hemoglobin at the end of the experimental period; HOMA-R, homeostasis model assessment; OB-A, non-diabetes with obesity group fed 0.05% α-tocopherol containing chow; OB-R, non-diabetes with obesity group fed 0.05% Ricetrienol-containing chow; OB-S, non-diabetes with obesity group fed standard chow; sBW, body weights at the start of the experimental period; TG-L, triglyceride contents of the liver tissue; NS, not significant. [a]$P < 0.001$, [b]$P < 0.01$, [c]$P < 0.05$ *vs* non-diabetes with obesity group fed standard chow; *$P < 0.001$, [#]$P < 0.01$, $P < 0.05$ *vs* obese type 2 diabetes group fed standard chow analyzed by Fisher's protected least significant difference test as a post-hoc test.

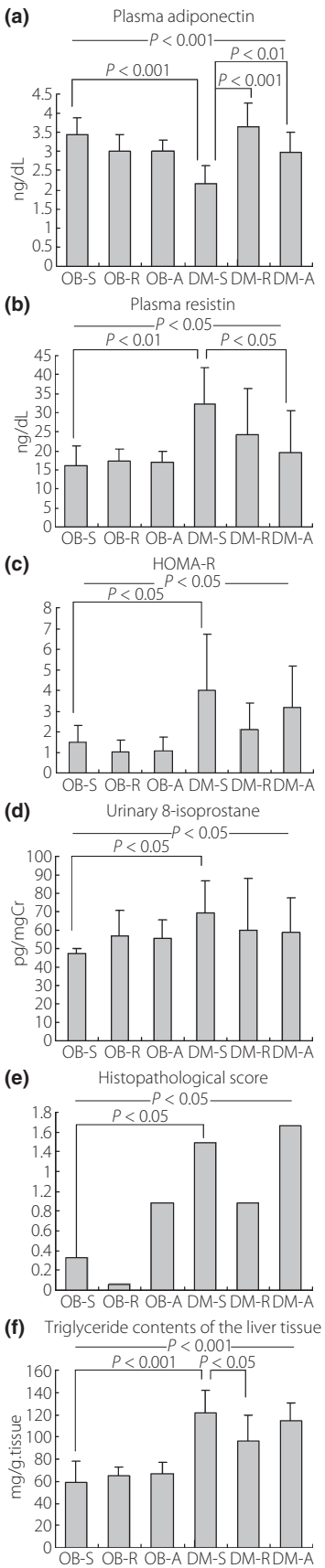

Figure 1 | Columns and error bar represent means and standard deviation, respectively. Histopathological scores were analyzed by the Kruskal–Wallis test for multiple comparisons and the Mann–Whitney *U*-test as a post hoc test. Other data were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test as a post-hoc test. (a) Plasma adiponectin was significantly reduced the obese type 2 diabetes group fed standard chow (DM-S) than the non-diabetes with obesity group fed standard chow (OB-S), but it increased significantly in the obese type 2 diabetes group fed 0.05% Ricetrienol-containing chow (DM-R) and the obese type 2 diabetes group fed 0.05% α-tocopherol containing chow (DM-A) compared to DM-S. (b) Plasma resistin increased significantly in DM-S as opposed to OB-S, but it was significantly reduced in DM-A compared with DM-S. (c) Homeostasis model assessment for insulin resistance (HOMA-R) was significantly elevated in DM-S compared with OB-S. (d) Urinary 8-isoprostane increased significantly in DM-S compared with OB-S. (e) The histopathological score was significantly elevated in DM-S compared with OB-S. (f) Triglyceride contents of the liver tissue were significantly increased in DM-S compared with OB-S, but they were significantly reduced in DM-R as opposed to DM-S.

all rats were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg bodyweight). Then, they were surgically opened and exsanguinated through the heart. Livers were removed and used for histopathological and chemical examination. All procedures were carried out in accordance with institutional guidelines for animal research.

Measurement of Glycated Hemoglobin, Plasma Insulin, Plasma Cytokines and Urinary 8-Isoprostane

Collected blood was heparinized and glycated hemoglobin (GHb) was measured by high performance liquid chromatography. Plasma insulin was assayed by enzyme immunoassay (EIA) using a rat insulin ELISA kit (Linco Research, St. Charles, IL, USA). Plasma adiponectin was assayed by EIA using a rat adiponectin ELISA kit (Otsuka Pharmaceutical). Plasma resistin was assayed by EIA using a rat resistin ELISA kit (BioVendor Laboratory Medicine, Brno, Czech republic). Plasma leptin was assayed by EIA using a rat leptin-HS ELISA kit (Yanaihara Laboratory, Shizuoka, Japan). Plasma monocyte chemoattractant protein-1 (MCP-1) was assayed by EIA using a rat MCP-1 ELISA kit (Endogen, Rockford, IL, USA). Plasma intercellular adhesion molecule-1 (ICAM-1) was assayed by EIA using a rat sICAM-1 (CD54) immunoassay (R&D Systems, Minneapolis, MN, USA). Urinary 8-isoprostane was measured by EIA using an 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI, USA).

Histopathological Examination and Liver Triglyceride Content

The removed livers were immediately fixed in a 10% phosphate buffered formalin solution and then embedded in paraffin. Samples were cut and stained with hematoxylin–eosin stain (HE) reagent for light microscopic observation. Histopathological findings were evaluated by scoring methods according

to the report of Kleiner *et al.*[18] through a microscope (Nikon, Tokyo, Japan) at ×100 and ×200 magnification. Steatosis was graded 0–3 according to the percentage of cells with fatty droplets (0, <5%; 1, 5–33%; 2, >33–66%; 3, >66%). Lobular inflammation was graded 0–3 according to overall assessment of all inflammatory foci (0, no foci; 1, <2 foci per ×200 field; 2, 2–4 foci per ×200 field; 3, >4 foci per ×200 field). Liver cell injury was graded 0–2 according to ballooning degeneration (0, none; 1, few balloon cells; 2, many cells/prominent ballooning). These three scores were added and the sum was used as a histopathological score. This assessment was carried out by three independent researchers who were unaware of the experimental conditions. Average values of the three mean scores assessed by these researchers were then used as a histopathological score for each animal. Histopathological scores for each rat were then collected and a comparison between groups was carried out by calculating the average histopathological score for each group.

Livers were also homogenized and the triglyceride contents of the liver tissue were determined by the Folch method.

Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Histopathological scores were analyzed by Kruskal–Wallis test for multiple comparisons and Mann–Whitney *U*-test as a post hoc test, as scores were not distributed normally. Other data were distributed normally, and were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test as a post-hoc test. *P*-values of <0.05 were defined as statistically significant.

RESULTS

Bodyweight, Blood Glucose, GHb, Plasma Insulin, Plasma Cytokines and Urinary 8-Isoprostane

Bodyweights, blood glucose, GHb and plasma insulin at the end of the experimental period are shown in Table 1. There was no significant difference in bodyweight between any of the groups. Although blood glucose levels and GHb were significantly higher in the DM group than the OB group, supplementation with RT or AT did not have any effect on blood glucose levels and GHb.

There was no significant difference in plasma insulin between any of the groups. Homeostasis model assessment for insulin resistance (HOMA-R = [plasma insulin] × [blood glucose]/405) was significantly higher in DM-S compared with OB-S (Table 1, Figure 1c). However, supplementation with RT or AT had no significant effect on HOMA-IR.

Plasma adiponectin was more significantly reduced in DM-S than in OB-S, but it increased significantly in DM-R and DM-A compared with DM-S (Table 1, Figure 1a). Plasma resistin increased significantly in DM-S as opposed to OB-S, but it was significantly reduced in DM-A. Plasma resistin was somewhat reduced in DM-R when compared with DM-S, but the difference was not significant (Table 1, Figure 1b). There was no significant difference in plasma leptin, MCP-1 or ICAM-1 in any of the groups (Table 1).

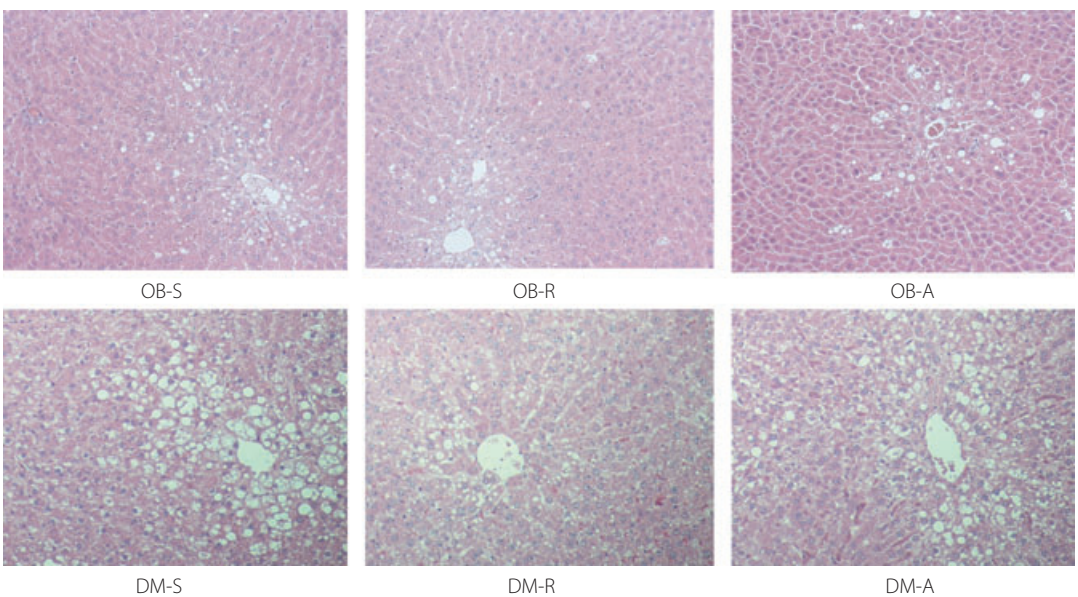

Figure 2 | Light microscopic photographs stained with hematoxylin–eosin reagent at ×200 magnification are shown. Fat deposits in the hepatocyte (vacuolated hepatocyte) were observed in all the groups in various degrees. These findings seemed to be less frequent and milder in the obese type 2 diabetes group fed 0.05% Ricetrienol-containing chow (DM-R) group compared with the obese type 2 diabetes group fed standard chow (DM-S) and obese type 2 diabetes group fed 0.05% α-tocopherol containing chow (DM-A) groups. OB-A, non-diabetes with obesity group fed 0.05% α-tocopherol containing chow; OB-R, non-diabetes with obesity group fed 0.05% Ricetrienol-containing chow; OB-S, non-diabetes with obesity group fed standard chow.

Urinary 8-isoprostane was converted into an index value based on creatinine per 1 mg of sample and the index of urinary 8-isoprostane increased significantly in DM-S compared with OB-S. Urinary 8-isoprostane was somewhat reduced in DM-R and DM-A as opposed to DM-S, but the difference was not significant (Table 1, Figure 1d).

Histopathological Examination and Liver Triglyceride Content

In histopathology, hepatic steatosis characterized by vacuolated hepatocyte was observed in all of the groups. These findings seemed to be less frequent and milder in the OB groups compared with the DM groups (Figure 2). Lobular inflammation and liver cell injury were very rare. Scores of three histopathological features (hepatic steatosis, lobular inflammation and liver cell injury) and overall histopathological scores are shown in Table 2. Hepatic steatosis and histopathological scores were significantly higher in DM-S compared with OB-S. The histopathological score was somewhat lower in DM-R than DM-S and DM-A, but the difference was not significant (Figures 1e and 2).

Although the triglyceride contents of the liver tissue increased significantly in DM-S compared with OB-S, they were significantly reduced in DM-R as opposed to DM-S (Table 1, Figure 1f).

Table 2 | Histopathological scores

	Hepatic steatosis	Lobular inflammation	Liver cell injury	Histopathological score
OB-S				
Mean	0.33	0.00	0.00	0.33
Range	0–1	0	0	0–1
OB-R				
M	0.06	0.00	0.00	0.06
Range	0–0.33	0	0	0–0.33
OB-A				
Mean	0.67	0.06	0.17	0.89
Range	0–1	0–0.33	0–1	0–1.33
DM-S				
Mean	1.17[c]	0.00	0.33	1.50[c]
Range	0.33–1.33	0	0–1	0.33–2.33
DM-R				
Mean	0.89	0.00	0.00	0.89
Range	0–1.66	0	0	0–1.67
DM-A				
Mean	1.28	0.00	0.39	1.66
Range	1–1.66	0	0–1	0–2.66
P-value	<0.05	NS	NS	<0.05

P-value indicates the statistical significance analyzed by the Kruskal–Wallis test. DM-A, obese type 2 diabetes group fed 0.05% α-tocopherol containing chow DM-R, obese type 2 diabetes group fed 0.05% Ricetrienol-containing chow; DM-S, obese type 2 diabetes group fed standard chow; OB-A, non-diabetes with obesity group fed 0.05% α-tocopherol containing chow; OB-R, non-diabetes with obesity group fed 0.05% Ricetrienol-containing chow; OB-S, non-diabetes with obesity group fed standard chow; NS, not significant. [c]$P < 0.05$ *vs* non-diabetes with obesity group fed standard chow analyzed by Mann–Whitney *U*-test as a post-hoc test.

DISCUSSION

The present study revealed the following four findings: (i) adipocytokine abnormalities, a reduction of plasma adiponectin and an increase of plasma resistin, were observed in non-treated diabetic OLETF rats, whereas these abnormalities could not be observed in non-diabetic OLETF rats; (ii) the administration of RT or AT significantly improved the reduction of plasma adiponectin, and the administration of AT significantly improved the elevation of plasma resistin in diabetic OLETF rats; (iii) hepatic steatosis (vacuolated hepatocyte) was observed in diabetic and non-diabetic OLETF rats. Histopathological scores of diabetic OLETF rats were significantly higher than those of non-diabetic OLETF rats, and the score tended to improve to some extent by the administration of RT; and (iv) the triglyceride content of the liver tissue in diabetic OLETF rats was higher compared with non-diabetic OLETF rats, and the administration of RT significantly reduced the accumulation of triglyceride in the liver.

Our first observation that adipocytokine abnormalities were found only in the diabetic rats might indicate that hyperglycemia plays a crucial role in the abnormal adipose tissue function. It is well known that adiponectin has an anti-atherosclerotic effect. Several studies have reported the reduction of plasma adiponectin in patients with macrovascular disease[19,20]. It has also been reported that overexpression of plasma adiponectin in apo-E KO mice improves atherosclerosis[21]. Resistin also has been reported to be involved in the development of atherosclerosis[22]. Therefore, an improvement of the adipocytokine abnormalities might reduce the frequency of macrovascular disease in type 2 diabetic patients.

Second, we showed that the administration of RT or AT improved the adipocytokine abnormalities in diabetic OLETF rats. As blood glucose levels and GHb were not reduced by the administration of RT or AT, the adipocytokine abnormalities were not improved by an amelioration of glycemic control. The present study showed a significant elevation of urinary 8-isoprostane in untreated diabetic OLETF rats compared with non-diabetic OLETF rats and that the administration of RT or AT reduced urinary 8-isoprostane in diabetes, but the difference was not significant. We have previously reported the anti-oxidative effect of RT in diabetic mice[15]. So, we think the anti-oxidative effect of RT or AT is a major mechanism for the improvement of adipocytokine abnormalities in diabetic rats, but further studies will be needed to elucidate the detailed mechanism.

Our third and fourth findings suggest the following idea. Although the fatty change of the liver was observed not only in diabetic but also non-diabetic obese rats, the severity of histopathological and histochemical change in the diabetic rats was more severe when compared with non-diabetic rats. It was therefore confirmed that the hyperglycemia was an exacerbation factor of a fatty liver. The administration of RT significantly

improved the fatty liver, whereas the administration of AT did not significantly improve the fatty liver in diabetic OLETF rats. The most plausible reason of fatty liver improvement by RT is an anti-oxidative effect, but other mechanisms should also be considered because of the poor effectiveness of AT (similar anti-oxidant to RT) administration. Another possible mechanism is an amelioration effect of adiponectin upregulated by RT. There are several reports that adiponectin had an improving effect to fatty liver[23,24], so the increase of adiponectin by RT might improve the fatty liver in diabetes. Another possible mechanism is the lipid lowering effects of RT administration. RT contains plant sterol, which suppresses cholesterol absorption in the intestine[25], so it might also improve the fatty liver. And, as RT is a mixture of multiple components, these other components might have had some effect.

Recently, the clinical usefulness of vitamin E administration on human non-alcoholic steatohepatitis has been reported[26]. The paper reported that vitamin E was more potent than pioglitazone in the prevention of steatohepatitis. RT was more effective than AT (vitamin E) in the present experiment. Thus, RT might be promising as a supplement for fatty liver prevention.

Considering all of these findings, the preventive effects of RT against adipocytokine abnormalities and fatty liver have been proven in diabetic OLETF rats. Therefore, RT might be a promising candidate as a supplement for the prevention of adipocytokine abnormalities and fatty liver in diabetic patients. It will be necessary to continue basic research to elucidate the pathophysiological mechanism of RT, and to evaluate clinical efficacy in a long-term, randomized, comparative trial.

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