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Studies on health beneficial functions of the enzymatically synthesized glycogen

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Studies on health beneficial functions of the enzymatically synthesized glycogen

(酵素合成グリコーゲンの健康機能に関する研究)

令和 元年 7月

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Abbreviation list

ESG; enzymatically synthesized glycogen

NO; nitric oxide

SCFAs; short chain fatty acids

NOG; natural origin glycogen

LDL; low-density lipoprotein

HDL; high-density lipoprotein

TG; triacylglycerol

NEFA; non-esterified fatty acids

ANOVA; analysis of variance

AUC; area under the curve

CT; computed tomography

TBA; total bile acid

PL; phospholipid

RT-PCR; real-time reverse transcription polymerase chain reaction

VLCAD; very-long-chain acyl-CoA dehydrogenase

LCA; long-chain acyl-CoA dehydrogenase

MCAD; medium-chain acyl-CoA dehydrogenase

FAS; fatty acid synthase

CPT; carnitine palmitoyltransferase

ACC; acetyl-CoA carboxylase

ACO; acyl-CoA oxidase

UCP; uncoupling protein

PPAR; peroxisome proliferator activated receptor

RPS17; ribosomal protein S17

VAS; visual analog scale

sIgA; secretory IgA

General introduction

The company name of Glico Co., Ltd. is derived from glycogen. Glycogen is a macromolecular glucan widely present in nature. Although the molecular weight of glycogen is large as 10^6 to 10^8 , the particle size of the molecule is about 20 to 60 nm in diameter, which indicate it has a very tightly packed structure [1]. Glycogen is present in animals, microorganisms and some plats and functions as an energy storage form. In animals, it is mainly contained in the skeletal muscle and liver. Glycogen plays as energy source for exercise in the skeletal muscle and regulating blood glucose level [2]. It is known that 5% of the liver and 0.5 to 1% of the muscle as wet weight correspond to glycogen. In addition, glycogen is known to exist in various tissues and cells such as brain, heart, leukocyte, skin, thymus, vagina, cartilage, etc. [3-9]. The role of glycogen is known as a storage of polysaccharide, but several reports have suggested other functions. For example, the antitumor effect of scallop and oyster derived glycogen has been reported. It was shown that intraperitoneally injection of glycogen significantly prolonged the survival days of the tumor transplanted mice [10]. However, studies on the role of glycogen other than energy storage source have been quite a few. Glycogen has been used for nourishment tonic and nutrition enhancement for a long time, and has been believed to be good for human health, although there was few data showing scientifically the health promotion effect of glycogen. Therefore, the author and collaborators have tried to develop the high purity and low cost glycogen synthesis method for elucidating the health promoting effect of glycogen.

In our laboratory, the author and the collaborators have been developed

carbohydrate-related enzymes and new methods of bioconversion of the carbohydrate by using the enzymes. In a few decades the author and the collaborators have launched some carbohydrate related new materials. For example, highly branched cyclic dextrin, which enhance endurance capacity during exercise by efficiently supplying energy [11]. Recently, the author and the collaborators have established the method to synthesize glycogen by using three enzymes [12], and named this product as 'enzymatically synthesized glycogen (ESG)'. In this production method, waxy corn starch is used as a raw material. First, α -1,6 bonds of waxy corn starch are completely cleaved by isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa* to produce short chain amylose. After heating to inactivate the enzyme, recombinant amylomaltase (EC 2.4.1.25) derived from Thermus aquaticus and branching enzyme (EC 2.4.1.18) from Aquifex aeolicus were added to the reaction mixture to efficiently synthesize glycogen. The collaborators confirmed glycogen obtained by this process has the same structure and properties as natural glycogen in various physicochemical properties such as chain length and degree of branching in the molecule, shape observation by electron microscope, intrinsic viscosity [13]. In addition, in order to use enzyme synthesized glycogen as a food material, the author and the collaborators considered that it is necessary to confirm safety of ESG, and carried out a safety evaluation test. Based on the results of the following three tests, it was concluded that ESG is safe as a food material. Single oral administration toxicity test in rat showed no toxicity by a dose of 2,000 mg / kg body weight. Thirteen week repeated feeding dietary toxicity test in rat indicated the safety at 20 g / kg body weight / day. Reverse mutation test using bacteria revealed no mutagenicity of ESG [14]. Based on these safety data, in accordance with the procedure prescribed by the US Food and Drug Administration, the author and the collaborators acquired GRAS (Generally

Recognized as safe) qualification in 2010. In Japan, the author and collaborators also obtained the permission from to Ministry of Health, Labor and Welfare that ESG can use as food ingredients.

Recent years, the author and the collaborators have tried to reveal the health promoting effect of ESG in cultured cells and rodent to recognize the function of glycogen. At first the immunomodulating action of ESG was studied in detail. The collaborators have obtained the following findings: (i) glycogen activates macrophages, which are important immune cells involved in antigen recognition, to produce nitric oxide (NO) and inflammatory cytokines such as interleukin-6 or interleukin-12 [15, 16], (ii) Toll-like receptor 2, which is a receptor involved in innate immunity, mediates the response of macrophages to glycogen [17], and (iii) orally administered ESG enhances systemic immune responses such as a tumor suppressor effect, activation of natural killer cells in the spleen, and suppression of splenic sympathetic nervous activity [18]. These results suggest that glycogen not only has a role in maintaining the blood glucose level, but also has immunomodulatory effects. However, how ESG is digested and absorbed when it is ingested has been unknown.

In this study, firstly, in order to confirm the digestibility of ESG in the intestinal tract, glycemic index of ESG in rats were measured by an oral glucose tolerance test. After that, the rats were fed foods of which carbohydrates were all replaced with ESG, and the amounts of glucan in the small intestine, cecal contents and feces were analyzed. As a result, it was revealed that about 20% of ESG reached the cecum as a large molecule glucan. Thereafter, the glucan from ESG was assimilated by intestinal bacteria, mainly *Lactobacillus* and *Bifidobacterium*, and was converted into short chain fatty acids (SCFAs). Unexpectedly, it was cleared that the body fat mass of rat fed ESG decreased.

Therefore, next, the author examined the anti-obesity effect of ESG. Rats were fed highfat diet containing ESG for 4 weeks. It was revealed that ESG significantly suppressed an increase in body fat compared with non-ESG treated the control, and ESG also decreased fat accumulation in the liver. On the one hand, body weight was not significantly changed by ESG. Analysis of feces revealed that the amount of fat excretion increased in ESG group. ESG promoted excretion of ingested fat into feces, while ESG did not affect lipid metabolism related mRNA expressions in the liver and adipose tissue.

To investigate the anti-obesity effect of ESG in more detail, a long-term experiment was carried out. A high-fat induced obese model of mice received 10 or 20% ESG for 15 weeks. As the result, suppression effect of ESG on body fat accumulation was confirmed. ESG also suppressed fat accumulation in the liver, and increased in cecal contents and excretion of lipids into feces. Although ESG did not affect the mRNA expressions of lipid metabolism related enzymes, the suppressive effect of ESG on lipid absorption was revealed by single oral administered experiment.

It was revealed that orally ingested ESG go through the small intestine partially with being digested and reach the cecum as macromolecules. As a result, ESG is expected to change the intestinal microbiota, strengthen intestinal tract immunity, and increase SCFAs to bring health benefits. Although it is necessary to obtain the evidence from a human clinical study in order to use as a functional ingredient of health-promoting food, ESG have not been revealed health benefit in a human study. The author and the collaborators tried human studies on the immunomodulating effect of ESG, but the results did not show clear evidence (unpublished data). The author considered to conduct a human clinical study to proof the effects on lipid absorbance or intestinal microbiota. However, in the market, there are so many functional foods claimed to reduce lipid absorption and improve intestinal microbiota. In recent years, it has been reported that strengthening the immunity and improving the intestinal environment enhance the cognitive function in the brain. It was reported that stimulation of innate immunity improves the memory function in Alzheimer's disease model mice [19]. In normal mice study, the improvement in the memory capacity thorough immunomodulation pathway associated with increase in IL-1 β , TNF- α , and IL-6 cytokines was reported. [20]. Wang *et al.* demonstrated that oligosaccharides from Liuwei Dihuang improve cognitive dysfunction via the improving in intestinal microbiome in the senescence-accelerated mouse-prone 8 [21]. Recent studies have demonstrated that prebiotic and indigestive polysaccharides show beneficial effects on the brain function. Polysaccharide extracted from Astragalus membranaceus was reported to attenuate cognitive disorder by affecting gut microbiota in diabetic mice [22]. Beta glucan was reported to enhance cognitive function through inhibition of acetylcholinesterase in scopolamine induced cognitive impaired rats [23], and reduce the number of degenerated neurons in the brain tissue of post-menopausal rats [24]. Therefore, the author hypothesized that ESG could improve cognitive function through immunomodulation and change in intestinal microbiota in human, and decided to conduct the clinical trial. In the randomized clinical study, the author divided 40 subjects randomly into 2 groups and ordered them to ingest 5 g of ESG or dextrin as placebo for 4 weeks, and then evaluated the brain function before and after ingestion. The test was carried out as a crossover trial. The results of the test demonstrated that an intake of ESG for 4 weeks improves visual discrimination ability and long-term memory ability. As well as Japan, it is clear that the aging society will advance in the world in the future, and it is expected that the number of patients with dementia will increase rapidly. Taken together the results of this study, the author have considered ESG could use as a functional food material, and

now the author and collaborators are planning to develop the health promoting products containing ESG.

Chapter 1

Metabolic fate of orally administered ESG in rats

Introduction

Some reports suggested that glycogen possessed the immunostimulating effects [10, 15 and 25], although the metabolic fate of orally administered glycogen have rarely investigated. In normal foods, glycogen is rich in the shellfish and animal livers. In addition, in several higher plants contain phytoglycogen, which is a glycogen-like polysaccharide [26]. The author and collaborators have developed industrial synthesis process of glycogen named it ESG [12]. It has been revealed that physicochemical parameters of ESG such as structural chain lengths, molecular shapes and so on are equivalent to these of natural origin glycogen (NOG) [12, 27]. The author and collaborators performed the digestive test of ESG *in vitro*. After digestion by an excess dose of α -amylase, about 40% of ESGs remained as a high molecular (> 1000 kDa) weight resistant fraction. On the other hand, the resistant fraction from NOGs were vary in content and size: resistant fraction from one NOG was high molecular weight, although 5 NOGs possessed the low molecular weight resistant fraction (< 10 kDa) in the previous study. It was evaluated the dietary fiber content of ESG was about 20% [13].

Many studies demonstrated beneficial physiological effects of dietary fiber in humans. For examples, water-soluble dietary fibers reduce the plasma low-density lipoprotein (LDL) cholesterol, on the one hand, some insoluble dietary fibers increase the bulk of feces [28, 29]. Animal and human studies have shown that oligofructose, inulin and resistant starch possess prebiotics function, which increase intestinal *Bifidobacterium* [30-32]. It has been suggested that appropriate fiber consumption could reduce the risk of cardiovascular diseases, colon cancer, and type II diabetes mellitus [33-35].

In this chapter, the author investigated the metabolic fate of ESG in rats by evaluating the glycemic index, intestinal fermentation and lipid metabolism. The effects of ESG and mussel glycogen, which was easily digested in previous *in vitro* test [13], were compared to the role of the high-molecular resistant fraction.

Materials and Methods

Materials

ESG was synthesized by the method described in general introduction and used in this study [12]. Mussel glycogen was purchased from Laboratories Serobiologique (Pulnoy, France). Table 1-1 shows the summary of molecular mass and resistant portion of ESG and NOG *in vitro* reported previously. Enzymes, α -Amylase, glucoamylase and isoamylase were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Toyobo Co. Ltd. (Osaka, Japan) and Hayashibara Biochemical Laboratories (Okayama, Japan) respectively, and used for analysis of the glycogen content in cecum and feces. All other chemicals were analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animal experiments

Animal experiments in this study were conducted in accordance with Guidelines of Science Council of Japan for Proper Conduct of Animal Experiments after approval of the Institutional Animal Care and Use Committee of Ezaki Glico Co., Ltd.. Sprague Dawley rats (male, 6w) were obtained from Japan SLC, Inc. (Shizuoka, Japan). Rats were acclimated to the environment for 1 week. Animal room was controlled in a temperature $(23 \pm 1^{\circ}C)$ and 12h light-dark cycle. Experiment 1: fourteen rats were divided into 2 groups of 7 each with approximately equal body weights, and rats were orally administered ESG or glucose as a control (2 g/kg body weight) by gavage after 18 h starvation. Before and after 10, 20, 30, and 60 min of the administration, blood was collected from the tail vein. The blood was centrifuged at 900 × g for 5 min to obtain plasma. Glucose and insulin concentrations in the plasma were measured with corresponding kit purchased from Wako Pure Chemical Industries and Shibayagi Co. Ltd.

(Gunma, Japan), respectively.

Experiment 2: rats were separated into 3 groups of 7 each with approximately equal average body weights. Rats were fed with the diets shown in Table 1-2 for 2 weeks. Every 2-3 days, the amount of food intake and body weight were measured. On day 10, fresh feces was immediately collected after excretion for analyzing fecal microbiota. On day 11 and 12, all feces were collected in order to determine α -glucan content. Rats were euthanized under anesthesia without fasting at the end of test. The liver, cecal content and epididymal adipose tissue were collected and weighed.

Experiment 3: twenty-one rats were separated into 3 groups of 7 each with approximately equal body weights to compare ESG and NOG. Control and ESG groups were fed the control and high-ESG diet shown in Table 1-2, and NOG group was fed the diet replaced ESG with glycogen from mussel for 2 weeks. The same parameters as in Experiment 2 were analyzed.

Experiment 4: fourteen rats were separated into 2 groups of 7 each with approximately equal body weights to evaluate the change in plasma lipid profile. Rats were fed the control or high-ESG diets shown in Table 1-2 for 2 weeks. After 6 h starvation, blood was collected from the heart under anesthesia. As the plasma lipids, triacylglycerol (TG), non-esterified fatty acids (NEFA), total cholesterol and high-density lipoprotein (HDL) cholesterol were measured by corresponding kit of Wako Pure Chemical Industries.

Glucose and α *-glucan analyses*

After lyophilized, cecal contents and feces were grinded and 100-200 mg were homogenized with PBS. The homogenate was centrifuged at $900 \times g$ for 10 min, then the supernatant was collect to measure the concentration of glucose. A 200-µl aliquot of the supernatant was treated with 30 U of isoamylase and 200 mU of α -amylase at 37°C for 2 h in 0.2 M acetic buffer at pH 5.5. After that 20 U of glucoamylase was added and incubated at 37°C for 16 h to evaluate the α -glucan concentration as a glucose concentration. The concentration of α -glucan was determined from the concentration of glucose of the reaction mixture and the untreated supernatant. The excretion rate of α -glucan in the feces was estimated from the values of total daily intake of α -glucan, total amount of daily feces and fecal concentration of α -glucan.

SCFAs analysis

Cecal content for GLC analysis was prepared according to the previous report to measure the SCFAs concentration [36]. One g of the homogenate of cecal content was adjust to pH 2.0 with 2N H₂SO₄ solution. After centrifugation at $6000 \times \text{g}$ for 15 min at 4°C, the supernatant was collected and filtered with a 5000 Da molecular-mass cut-off membrane (Vivascience AG, Hannover, Germany) by centrifugation at $8000 \times \text{g}$ for 1 h at 4°C. The concentrations of SCFAs of the filtrate were measured by GLC: Shimadzu GC-2014 (Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector and a Thermon-3000 glass column packed with Chromosorb W (Shimadzu Co.). The carrier gas was nitrogen and a flow rate was 50 ml/min. The temperature of the injection port and detector was 250°C. The temperature of column oven was kept at 130°C for 13 min, then heated to 240°C for 6 min to remove other organic compounds

Measurement of fecal microbiota

One g of fresh feces was suspended in PBS containing 0.05% (w/v) cysteine-HCl and 0.1% (w/v) purified agar to analyze a fecal microbiota. Six intestinal microorganisms, *Bifidobacterium, Lactobacillus, Bacteroidaceae, Staphylococcus, Streptococcus,* and *Enterobacteriaceae* were analyzed by the method reported by Ikeda *et al.* [37]. *Statistical analysis*

All data in this experiment are expressed as mean \pm SD. Student's *t*-test was used to analyze the difference between two data. The comparisons of three data were analyzed by one-way analysis of variance (ANOVA) and followed by a post-hoc Fisher's test with Stat View (Abacus Concepts, Berkeley, CA). Statistically significance means *p*-values were <0.05.

Results

Single oral administration test

The concentrations of glucose and insulin in plasma after administration of ESG or glucose were shown in Fig. 1-1. The increase in the plasma glucose level of ESG group was significantly lower at 20 and 30 min than that of glucose-given control (Fig. 1-1A). Glycemic index calculated from the area under the curve (AUC) of glucose was 79.0 in ESG group (Fig. 1-1B). The result of insulin level was similar to the glucose level in both groups, although significant difference was not observed (Fig. 1-1C).

Effects of 2-week ingestion of ESG

In the high-ESG group, body weight gain was significantly lower than those of the other groups after 2-week (Table 1-3). Although significant difference was not observed, in the high-ESG group the amount of food intake was slightly lower than that in the control group. In the high-ESG group, epididymal fat weight was 81.6% of the control group with a significant difference. Weight of the liver was not changed in any group (Table 1-3). In the high-ESG group, the cecal content significantly increased (Fig. 1-2A). Cecal content pH in both the high-ESG and the low-ESG groups were significantly lowered compared with that in the control group (Fig. 1-2B). The result of the analysis of fecal microbes shown in Table 1-4. In the high-ESG group, the numbers of

Bifidobacterium and *Lactobacillus* were significantly increased than those of the control group. The amounts of acetate, propionate, butyrate and total SCFAs were significantly increased in the high-ESG group. Total amount of SCFAs of the low-ESG group was significantly increased than that in the control group (Fig. 1-3). The result indicated that indigestible portion of ESG reach the cecum. Thus, the author next examine the glucose and α -glucan concentrations in the cecal content and feces. In the high-ESG group, the α -glucan concentration of the cecal content was significantly higher than that of the control group, while no significant change was observed in the low-ESG group (Table 1-5). As the result of the cecal content, glucose concentration was significantly higher than that of the control group in both ESG groups. In the high-ESG group, α -glucan and glucose concentrations in the feces were significantly increased compared with those of the control group. The author estimated the ratios of the excretion amount of α -glucan to the intake amount were 0.06%, 0.48% and 0.13% in the control, the high-ESG and the low ESG groups, respectively.

Comparison of ESG and NOG

Next, the author substituted NOG for ESG in high-ESG diet to evaluate the relation between the molecular size of the resistant fraction and dietary fiber-like effect. As the results, ESG significantly affected the amount and pH value of the cecal content, while NOG did not altered both parameters compared with those of the control group (Fig 1-4A, B). In NOG group, there were no significant change in the α -glucan concentration, SCFAs production and weight of the epididymal fat (Table 1-6).

Plasma lipid profiles

Finally, the author evaluated the changes in the plasma lipids by ESG supplementation. ESG significantly reduced the concentrations of plasma TG and total cholesterol as compared to those of the control group (Table 1-7). In the high-ESG group, the ratio of HDL/total cholesterol was significantly increased as compared to that of control group, although plasma HDL-cholesterol concentration was significantly decreased. ESG did not change the concentration of NEFA.

Discussion

The author investigated the metabolism of ESG after oral administration to rats. The result indicated that ESG's glycemic index was 79 (Table 1-1), and this value was similar to dietary fiber *in vitro* [13]. It is suggested that the enzymes in the intestinal surface [38] work to digest approximately 80% of ESG to glucose, while the detail of the resistant portion of ESG is unknown. After the digestion of ESG by α -amylase, about 40% remained as a high molecular (>1000 kDa) resistant fraction (α -macrodextrin) *in vitro* study (Table 1-1) [13]. It is hypothesized that the structure of resistant portion of ESG *in vivo* has been similar to the characteristics of highly branched revealed *in vitro* study. The results of this study indicated that the α -macrodextrin from ESG was utilized by *Bifidobacterium* and *Lactobacillus* to produce SCFAs. Few amounts of α -macrodextrin (<0.5% of ingestion) was observed in the feces.

The author compared the metabolic fate of ESG and NOG from mussel, which showed the smallest resistant fraction in an *in vitro* study [13]. Changes in the SCFAs production, cecal glucan content and epididymal fat weight observed in the ESG group was not shown in the NOG group (Table 1-6). Although the physicochemical properties of ESGs was found to be equivalent to NOGs, slight difference in the fine structures between ESGs and NOGs was suggested. Briefly, the distribution of α -1,6-branches in ESG shows more simple than that in NOG [13]. It is suggested that the branches in ESG molecule could not be attacked by α -amylase because of spans between branches should be 1 or 2 glucose units [13]. The spans between branches in NOG should be more varied (0-4 units) that those of ESG, thus some parts of the branches which has more than three glucose span could be degraded by α -amylase. This difference in the molecules should contribute with the difference between ESGs and NOGs in the resistant properties. Since NOGs with resistance activity to α -amylase is possible to have similarly effect with ESG, thus studies on the effects of NOG, which has high molecular resistant portion, could be interesting study.

The author demonstrated that ESG decreased the plasma TG and total cholesterol levels and epididymal fat weight. On the one hand, ESG increased the ratio of HDL/total cholesterol in this study. These results were similar to the effects of dietary fibers. The beneficial effects of dietary fibers on lipid metabolism have been shown to be varied: for instance, some soluble dietary fibers have been reported to reduce the blood cholesterol and the liver cholesterol in cholesterol-fed rats without change in the TG level [39]. The effects of appropriate soluble dietary fibers on lowering the concentration of LDL and total cholesterol in blood without affecting the concentration of HDL and TG have been shown in the epidemiological and intervention studies [28, 33, 40, and 41]. On the one hand, it has been reported that the concentrations of TG and total cholesterol were decreased by resistant starch with reduction in epididymal adipose tissue in a rodent study [42]. Resistant starch rich potato pulp is also reported to reduce the concentrations of blood cholesterol and TG [43]. Plasma cholesterol as well as TG concentrations were reduced by inulin and inulin-type fructans through the suppression of the lipogenesis in the liver [44]. Michael J. K. et al. reported that abdominal fat accumulation reduced by supplementation of resistant starch in rats [45]. The viscosity and/or colonic fermentation

should be related to the effects of dietary fibers. Therefore, differences in colonic fermentation of dietary fibers should related to the difference of the effects. Inulin, inulintype fructans and resistant starch have been known as prebiotics [30-32], which possess beneficial effect on the host through the growth of intestinal bacteria such as *Bifidobacterium* [46]. In this study, *Bifidobacterium* and *Lactobacillus* were significantly increased by ESG supplementation in the intestine. The effects of ESG on lipid metabolism and microbiota were similar to those of inulin, inulin-type fructans and resistant starch. The diet used in many studies that evaluate the effect of dietary fiber on lipid metabolism contains 5–10% of fiber, while the author used ESG at 63 and 17.8% in this study. The contents could be converted to 12 and 3.5% as the fiber, respectively, because ESG contains about 20% dietary-fiber like portion. Therefore, the findings in this chapter could be comparable with those of previous studies used dietary fiber. On the other hand, lipid metabolism was not affected by NOG from mussel (data not shown). The author considered that the beneficial effects on lipid metabolism is related to the high molecular resistant fraction of ESG.

ESG ingestion greatly increased production of SCFAs in the cecum (Fig. 1-3). It has been reported that acetate possesses beneficial effects on energy metabolism [47-49]. Propionate is reported to inhibit the acetate transportation into hepatocytes, although acetate is substrate of lipogenesis in hepatocytes [50]. From the view point of cholesterol metabolism, acetate is known as a substrate, while propionate inhibits cholesterol synthesis [51]. Therefore, the increase in propionate/acetate ratio could result in the reduction of the plasma cholesterol and TG levels. The approximate molar ratio of cecal acetate/propionate/butyrate is reported to 65:25:10 in rats fed a normal diet [52]. The ratio of acetate/propionate/butyrate in cecal content in the high- and low-ESG groups were 48:26:26 and 60:18:22, respectively, while those in the control group were about 70:20:10, in this chapter. These results indicated that both the increase in SCFAs quantities and the change in the ratio related to changes in the lipid metabolism.

Recently, it is reported that leptin production in adipocytes is stimulated with SCFAs through GPR41, G protein-coupled receptor, and supplementation of propionate in mice increases plasma leptin level [53]. It is well-known that leptin is anti-obesity hormone secreting from adipose tissue and known as the inhibitor of energy intake and the energy consumption enhancer [54]. The food intake was not affected by ESG, although total production of SCFAs was significantly increased by 3-folds in the high-ESG group in this chapter. This could be explained by the reduced volume of the subcutaneous adipose tissue as the leptin secretion organ [54]. Otherwise, adipogenesis of 3T3-L1 cells is stimulated by acetate and propionate [55]. Further study is needed to investigate the mechanism of the anti-obesity effect of ESG.

	Initial M _w	M_w of 24-h α -amylase digests (%)			Dietary fiber
		>10 k	1–10k	<1 k	content (%)
ESG	7000 k	38.2	N.D.*	61.8	22.1
NOG from mussel**	5100 k	N.D.	43.3	56.6	< 0.2

Table 1-1 Comparisons of initial and α -amylase digests' M_w and dietary fiber content between ESG and NOG

* N.D.: Not detected,

** Data from Ref [13].

	Diet group		
	Control	High-ESG	Low-ESG
		g/100 g	
α-Corn starch	63	-	47.2
ESG	-	63	15.8
Casein	20	20	20
Corn oil	5	5	5
Cellulose	5	5	5
Mineral mix *	5	5	5
Vitamin mix *	2	2	2
Calorie (kcal/100g)	377	377	377

Table 1-2 Composition of the experimental diets

* AIN93 composition obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan).

	Diet group		
	Control	High-ESG	Low-ESG
Body weight gain (g)	95.4 ± 8.4 $^{\rm b}$	85.1 ± 9.6 a	96.7 ± 8.5 $^{\rm b}$
Food intake (g/day/7 rats)	109 ± 10	103 ± 12	111 ± 9
Organ weight (% of body weight)			
Liver	4.06 ± 0.38	4.12 ± 0.25	4.20 ± 0.38
Epididymal fat	1.41 ± 0.25 $^{\rm b}$	1.15 ± 0.17 a	1.42 ± 0.20 b

 Table 1-3
 Body weight gain, food intake, and organ weights

Values are mean \pm S.D; n = 7.

Different superscript letters mean significantly different (p < 0.05).

Table 1-4 Effects of ESG on gut microbiota

		Diet group		
	Control High-ESG Low-ES			
		log ₁₀ /g wet feces		
Bifidobacterium	7.1 ± 0.66 $^{\rm a}$	10.7 ± 0.25 $^{\rm b}$	7.8 ± 1.30 ^a	
Lactobacillus	7.6 ± 0.43 $^{\rm a}$	9.1 ± 0.45 $^{\rm b}$	8.4 ± 0.43 $^{\rm a}$	
Enterobacteriaceae	6.8 ± 0.66	6.4 ± 0.43	6.4 ± 0.43	
Streptococcus	7.4 ± 0.71	6.7 ± 0.57	7.1 ± 0.45	
Staphylococcus	6.0 ± 0.44	4.4 ± 0.92	5.9 ± 0.60	
Bacteroidaceae	9.5 ± 0.48	9.7 ± 0.52	9.5 ± 0.21	

Values are mean \pm S.D; n = 7.

Different superscript letters mean significantly different (p < 0.05).

	Diet group		
	Control	High-ESG	Low-ESG
	mg/g cecal content		
α-Glucan	0.77 ± 0.50 $^{\rm a}$	8.0 ± 7.8 ^b	2.0 ± 1.9 ^a
Glucose	3.6 ± 2.4^{a}	$9.1\pm5.0~^{\rm b}$	9.6 ± 5.7 b
		mg/g feces	
α-Glucan	2.7 ± 1.7 $^{\rm a}$	$23.7\pm12.6\ ^{\text{b}}$	7.2 ± 2.5 ^a
Glucose	3.6 ± 3.0 ^a	$8.8\pm1.0\ ^{b}$	2.8 ± 2.1 a

 Table 1-5
 concentrations of α-glucan and glucose in cecal content and feces

Values are mean \pm S.D; n = 7.

Different superscript letters mean significantly different (p < 0.05).

weight			
	Diet group		
	Control	ESG	NOG
Cecal SCFA production			
(µmol/cecum)			
Acetate	48.2 ± 24.1 $^{\rm a}$	230.1 ± 139.2 $^{\rm b}$	$38.1 \pm 11.4 \ ^{\rm a}$
Propionate	16.0 ± 6.2 $^{\rm a}$	80.4 ± 43.6 $^{\rm b}$	14.5 ± 3.5 $^{\rm a}$
Butyrate	10.1 ± 4.6 $^{\rm a}$	62.0 ± 29.0 $^{\rm b}$	14.5 ± 3.5 $^{\rm a}$
Cecal α-glucan			
(mg/g cecal content)	0.54 ± 0.16 a	5.42 ± 7.12 $^{\rm b}$	0.28 ± 0.20 a
Epididymal fat (g)	1.6 ± 0.2 $^{\rm a}$	1.1 ± 0.1 b	1.6 ± 0.3 a

Table 1-6 SCFA production, α -glucan concentration in the cecum, and epididymal fat weight

Values are mean \pm S.D; n = 7.

Different superscript letters mean significantly different (p < 0.05).

	Diet group		
	Control	High-ESG	
TG (mg/dL)	86.2 ± 15.7	$51.8 \pm 11.7^{**}$	
Cholesterol			
Total (mg/dL)	62.2 ± 5.5	$47.2 \pm 4.2^{**}$	
HDL (mg/dL)	42.1 ± 3.1	$34.2 \pm 3.3^{**}$	
HDL/total ratio (%)	67.8 ± 3.0	$72.5\pm2.8^*$	
NEFA (mEq/L)	0.68 ± 0.19	0.53 ± 0.12	

 Table 1-7
 Effect of glycogen on the plasma lipid levels

Values are mean \pm S.D; n = 7.

*,** Mean values were significantly different from those of control group by Student's *t*-test (* p < 0.05, ** p < 0.01)



Fig. 1-1 The change in plasma glucose and insulin concentration in ESG-treated rats. Glucose (control) or ESG were administered to rat with dosage of 2 g/kg body weight. Before and 10, 20, 30, and 60 min after administration, blood samples were collected from tail vein. Glucose (A) and insulin (C) were measured and expressed as closed circles (control) and squares (ESG), respectively. B shows the glycemic index calculated from data. Values are mean \pm S.D., n = 7. * shows significant difference from the control group.



Fig. 1-2 The change in body weight and pH value of cecal content by ESG ingestion. Rats were fed the diets indicated in Table 2 for 2 weeks. The weight (A) and pH (B) of the cecal content were measured and shown. Values are mean \pm S.D., n = 7. Different letters in the bar indicate significant difference from each other.



Fig. 1-3 ESG ingestion increased the amounts of cecal SCFAs. Rats were fed the diets indicated in Table 2 for 2 weeks. The amounts of the SCFAs were measured by GLC analysis in the cecal content and shown. The total SCFA amount means the sum of the amounts of acetate, propionate and butyrate. Values are mean \pm S.D., n = 7. Different letters in the bar indicate significant difference from each other.



Fig. 1-4 ESG but not NOG ingestion altered weight and pH value of cecal content. Rats were fed the diets indicated in Table 2 for 2 weeks. The weight (A) and pH (B) of the cecal content were measured and shown. Values are mean \pm S.D., n = 7. Different letters in the bar indicate significant difference from each other.

Chapter 2

ESG reduces lipid accumulation in diet-induced obese rats

Introduction

As described in Chapter 1, indigestible fraction of ESG work as dietary fiber when it is administrated to rats. ESG reach the cecum with high molecular and is used by the intestinal microbiome to produce SCFAs [56]. It has been well known that dietary fibers have beneficial effects on lipid metabolism [57, 58]. In Chapter 1, the author demonstrated that ESG decreased plasma TG concentration and epididymal fat weight, and increased the ratio of HDL/total cholesterol in normal rats [56] although, the detail in the mechanism of the effects are unclear.

In the world, obesity has been considered as health and social problems [59]. Many studies have been performed to prevention of obesity, in these decades [60-62], although the problem has not diminished at all. The definition of obesity is an excessive accumulation of lipid in the adipose tissues which is caused by the energy consumption and expenditure imbalance [59]. It is important to clarify the antiobesity effect of food factors to understand and reduce human obesity problem. It is hypothesized that supplementation of ESG could prevent diet-induced obesity, because ingestion of ESG reduced plasma lipids and fat accumulation in normal rats. To clarify this hypothesis, the effects of ESG on fat accumulation and plasma lipid profiles, lipid excretion in the feces and the lipid metabolism related mRNA expression levels were investigated in a diet-induced obese rat model.

Materials & Methods

Materials

In this chapter, the author used ESG which was synthesized as described in general introduction [12]. The diets for animal study and all other chemicals were obtained from Research Diets, Inc. (New Brunswick, NJ) and Wako Pure Chemical Industries, respectively.

Animal experiments

The animal experiment was performed after approval by the Institutional Animal Care and Use Committee (Permission number: 21-07-01) in Kobe University. The experiment was carried out according to the Animal Experimentation Regulations of Kobe University. Sprague Dawley rats (3-weekold, male) obtained from Japan SLC, Inc. were acclimatized in an animal room (25 \pm 3°C, 60 \pm 5% humidity) for one week. After that, 30 rats were divided into 4 groups; control (C) group (n = 8), control-ESG20% (C20%) group (n = 7), high-fat (HF) group (n = 8), and high-fat-ESG20% (HF20%) group (n = 7) and were fed the diet shown in Table 2-1 for 4 weeks.

The author measured the body weight and food intake once a week. At third week, the body composition was analyzed by an X-ray computed tomography (CT) scanner for animal experimental use, the Latheta LCT-100 (Hitachi LTD., Tokyo, Japan) under anesthetized with isoflurane. CT images obtained from the diaphragm to the tail at 1 mm intervals were quantitatively assessed by using the Latheta software, version 2.10, to analyze the visceral and subcutaneous fat mass. At the last day, after a 14-h fasting, blood was collected from cardiac puncture then the rats were euthanized under anesthetized with sodium pentobarbital. The liver, muscles, mesenteric, epididymal, and perinephric adipose tissues, and cecum were weighed and stocked at -80°C.

Measurement of blood parameters

To obtain plasma, the blood was centrifuged at $9700 \times g$ for 10 min immediately and plasma was collected and stocked at -80° C. The author measured the plasma level of glucose, TG, total cholesterol, total bile acid (TBA) and NEFA, by using LabAssayTM glucose, Triglyceride-E test WAKO, LabAssayTM cholesterol, TBA test WAKO and NEFA-C test WAKO (Wako Pure Chemical Industries, Ltd.), respectively. Insulin and adiponectin concentrations were measured by ELISA kits (Shibayagi Co., Ltd.).

Measurement of lipid levels on the liver and feces

Lipids were extracted from the liver and feces by the method of Folch *et al.* [63]. Briefly, approximately 100 mg of samples, which were lyophilized and ground, were homogenized in 350 μ l of distilled water, then mixed with 700 μ l of chloroform/methanol (2/1, v/v) to extract lipid. After three times extraction, collected chloroform layers were mixed with quarter of KCl solution (0.88%, w/v), and vortexed. Then, the chloroform layer was collected after centrifugation (1800 \times g, 10 min), and evaporated and weighed to measure total lipid. The residue was dissolved in isopropanol with 10% (v/v) Triton-X, and total cholesterol, TG and phospholipid (PL) concentrations were measured by assay kits (Wako Pure Chemical Industries). TBA test WAKO was used to measure the TBA concentration of lipid from the fecal.

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Sepazol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan) was used to extract total RNA from the skeletal muscle, liver and adipose tissue. ReverTra Ace[®] qPCR RT Kit purchased from Toyobo Co., Ltd. (Osaka, Japan) was used to synthesize first-strand cDNA from 0.15 μ g of DNase I treated RNA with random primers. cDNAs of very-long-chain acyl-CoA dehydrogenase (VLCAD), long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), fatty acid synthase (FAS), carnitine palmitoyltransferase 1a (CPT1a), carnitine palmitoyltransferase 1b (CPT1b), acetyl-CoA carboxylase α (ACC α), acetyl-CoA carboxylase β (ACC β), acyl-CoA oxidase (ACO), uncoupling protein 1 (UCP1), uncoupling protein 2 (UCP2), uncoupling protein 3 (UCP3), peroxisome proliferator activated receptor α (PPAR α), peroxisome proliferator activated

receptor δ (PPAR δ), adiponectin, leptin and ribosomal protein S17 mRNA (RPS17) as the internal standard were amplified with the primers indicated in Table 2-2. Expression of mRNA was quantified in duplicate by the Real-Time PCR system (Applied Biosystems 7300) using THUNDERBIRDTM SYBR[®] qPCR Mix (purchased from Toyobo Co., Ltd.).

Statistical analysis

All data are shown as means \pm S.E. The significance analysis was performed by one-way ANOVA and followed by a post-hoc Tukey-Kramer test. When *p* values were < 0.05, the difference was considered as significant.

Results

Body weight and food intake

In the HF group, the body weight significantly increased as compared to the other groups from second week to the end of the experiment (Table 2-3). No significant change was shown in food intake and food efficiency among the groups (Table 2-3).

Lipid accumulation in the adipose tissue

When CT analysis performed on the third week, the amount of total fat was significantly higher in the HF group than that of the other groups (Fig. 2-1A). The result in subcutaneous and visceral fat masses was similar with total fat mass (Fig. 2-1B, C). One of the images in each group were exemplified in Fig. 2-1D. In the images, muscle or viscera and adipose tissue were indicated as gray and black parts, respectively. The percentages of the visceral adipose tissues, liver, cecum and pancreas to the body weight were shown in Table 2-4. The lipid accumulations in the mesenteric and perinephric adipose tissue by a high-fat diet were significantly suppressed by ESG supplementation. This result was similar to that from the CT
analysis. In the ESG groups, the weight of cecum and the amount of cecal content were significantly higher than those of control groups.

The liver lipids

The contents of total lipid, total cholesterol, TG and PL was significantly increased by high-fat diet in the liver, while the increase in the total lipid and TG was significantly reduced by ESG (Fig. 2-2A-D).

Plasma parameters

The plasma concentrations of glucose, insulin and total cholesterol were neither changed by a high-fat diet nor ESG (Table 2-5). In the C and C20% groups, the concentration of plasma TG was significantly higher than that in the HF and HF20% groups (Table 2-5). The level of NEFA was significantly lowered by a highfat diet (Table 2-5). The concentration of plasma TBA was significantly higher in the HF20% group than that in the C and HF groups. The lower adiponectin concentration was observed in the HF20% group as compared to other groups (Table 2-5).

Fecal lipid

ESG significantly increased fecal TG content in both normal and high-fat diet given animals (Fig. 2-3A). ESG also significantly increased the excretion amounts of total cholesterol and PL to the feces (Fig. 2-3B, C). Fecal TBA content in the C20% group was significantly higher than that of other groups (Fig. 2-3D).

Expression of Lipid metabolism-related mRNA

In the liver, the mRNA expression of ACO, ACCa, ACCb, and FAS was significantly decreased by high-fat diet (Fig. 2-4A), while that of CPT1A, LCAD, and UCP2 were neither altered by high-fat diet nor ESG. In the muscle, the expression of LCAD and MCAD mRNA were significantly increased in the HF20% group compared to the HF group, although no significant change was observed in CPT1B, VLCAD, and ACO, (Fig. 2-4B). PPARα, PPARδ or UCP2 mRNA expressions were not changed by high-fat diet and ESG supplementation (Fig. 2-4B). The mRNA level of UCP2 in the adipose tissue was significantly lower in the HF group than that in the C group, although there was no change in UCP1 and UCP3 among all groups (Fig. 2-4C). The expression of mRNA of adiponectin was significantly decreased by high-fat diet compared to that of the C group (Fig. 2-4C), while mRNA level of leptin was not changed in all groups (Fig. 2-4C).

Discussion

The lipid accumulation in the subcutaneous and visceral adipose tissues and liver were significantly increased (Table 2-4 and Figs. 2-1 and 2-2), although the plasma TG concentration in the HF group was lower than that in the C group (Table 2-5). The length of feeding period in this experiment could be too short to induce hyperlipidemia. In some reports, the hyperlipidemia is induced by a high-fat diet consumption more than 10 weeks [64-66]. It is considered that this study is the early stage model of obesity in which prior to the increase in the plasma lipids. It is possible that the increase in lipid uptake to the adipose tissue is the reason of the decrease in the plasma TG induced by high-fat diet. If the experimental period is longer, the blood lipid levels should be higher, because lipid accumulation of the adipose tissue would flood to blood.

In this chapter, high-fat diet induced lipid accumulation in the subcutaneous and visceral adipose tissues was suppressed by ESG supplementation (Table 2-4). The total lipid and TG contents in the liver were also suppressed by ESG (Fig. 2-2). The increase in lipids excretion to the feces could partly explain the reduction in those accumulation in the liver and adipose tissue. In Chapter 1, the author demonstrated that ESG possess about 20% of dietary fiber like portion [56]. It is well known that dietary fibers enhance the lipid excretion to the feces [67, 68]. The author considered that ESG act like a 'dietary fiber', because ESG consumption increased the cecal weight and content in this study.

Lipids excretion to feces in the HF20% group was significantly enhanced by EGS (Fig.2-3). When compared to the HF group, the increased amounts of TG, total cholesterol and PL were 0.9, 3.8, and 1.0 mg / g feces, respectively, and it was calculated to be 2.7 mg, 11.4 mg, and 3 mg per day, respectively. Total amount of increase in the lipid excretion in the feces could estimate to 0.5 g in 4 weeks, while the lipid accumulation suppressed by ESG in the perinephric, mesenteric and epididymal adipose tissues were about 1.9, 1.2 and 1.1 g, respectively. Thus, it is suggested that there are other mechanisms related to the effect of ESG on lipid accumulation in the liver and adipose tissue. From the results in this chapter, two possible mechanisms are suggested. The increase in fatty acid oxidation related mRNA expression in the muscle and the increase in the plasma TBA could be the mechanism of the anti-obese effects of ESG.

It is reported that oral supplementation of cholic acid, suppress accumulation of TG in the liver, VLDL secretion, and serum TG elevation in a hypertriglyceridemia mice model [69]. In addition, the plasma concentration of TBA and the energy consumption in the brown adipose tissue were increased by bile acids in mice, and prevention of obesity and insulin resistance was observed by bile acid administration [70]. Thus, it is possible that reduction of lipid accumulation in the adipose tissue and liver in the HF20% group was partly caused by the increased plasma TBA concentration.

The lipid metabolism related to mRNA expressions in the liver and adipose tissue were not affected by ESG. Thus, the author considered ESG did not alter lipid metabolism in mRNA level. On the one hand, in the present study, the mRNA levels of mitochondrial fatty acid oxidation related enzymes, LCAD and MCAD, in the muscle was increased by ESG under the high-fat diet condition. The skeletal muscle is related to 20–30 % of energy expenditure at rest, thus the skeletal muscle fatty acid oxidation is important for the energy expenditure [71]. Recently, it have been reported that the dysfunction of mitochondrial in the skeletal muscle linked with the risk of obesity and type 2 diabetes [72-74]. In the mice knocked out acetyl–CoA carboxylase 2, continuous oxidation of fat is observed, the total energy expenditure is increased, and insulin sensitivity was improved [75]. Increases in hepatic lipid and body fat accumulation were enhanced by mitochondrial dysfunction resulted in LCAD deficiency [76]. The upregulation of LCAD and MCAD, therefore, could be one of the mechanism of anti-obese effect of ESG through energy metabolism.

The author have two hypothesis related to the effect of ESG on increases in the muscular gene expression and plasma TBA. One is that the effects of ESG express through a stimulation to the cells in the small intestine. Kakutani *et al.* reported that the function of the intestinal immune system was enhanced by orally administrated ESG through a signal transmission in the small intestine [18]. Second, SCFAs could affect to the muscle and/or liver as a signal transducer. The author have already shown in Chapter 1 that SCFAs production in cecum is increased by the administration of ESG in rat [56].

In conclusion, the author revealed the anti-obese effect of ESG. Understanding the food factors possess anti-obesity effects could help us to prevent from obese, although this chapter provides only limited understanding of anti-obese effect of ESG. Thus, to clarify more detail of anti-obesity effect of ESG and to use ESG for health promotion food, further studies in rodent with long period and human clinical trials are necessary.

	Diet group					
	С	C20%	HF	HF20%		
		g / 1000g				
Casein	190	190	258	258		
L-Cystine	2.8	2.8	3.9	3.9		
Corn starch	299	133	0	0		
Maltodextrin	33	33	162	0		
Sucrose	332	332	89	89		
ESG	0	200	0	200		
Sovhean oil	24	24	32	32		
Lard	19	19	317	317		
Cellulose	47	13	65	26		
Mineral mix S10026	9.5	9.5	13	13		
Dicalcium phosphate	12	12	17	17		
Calcium carbonate	5.2	5.2	7.1	7.1		
Potassium citrate	16	16	21	21		
Vitamin mix V10001	9.5	9.5	13	13		
Choline bitartrate	1.9	1.9	2.6	2.6		
	(k cal / 1000 σ)					
Calorie	3845	3846	5242	5261		

Table 2-1 Diet composition and calorie of each group*

* All diets were purchased from Research diets, Inc.

target	sense	antisense
ΑССα	CAA CCA CTA CGG CAT GAC TCA	AGG TGG TGT GAA GGC GTT GT
ΑССβ	CTC ATC GCC AAT AAT GGT ATC G	GCG CGT TCA TTA CGG AAC AT
FAS	CAG CTG TCA GTG TAA AGA AAC ATG TC	AGC TCA CGT GCA GTT TAA TTG TG
CPT1A	CAT TAC AAG GAC ATG GGC AAG TT	CGG AAG AGC CGG GTC AT
CPT1B	TTC CGG GAC AAA GGC AAG T	CCT CTC GGA ACA TTC TTG TCA TG
ACO	TTT GTT GTC CCT ACT CGT GAA A	CCG ATA TCC CCG ACA GTG AT
VLCAD	TGC CAG GGC GGA ATC TAA G	GGT GGT AAG CTG GCC TTT GA
LCAD	TTG CAA ATT ACG GCA CAA AAG A	TGC CCG CCG TCA TCT G
MCAD	TTT CGG AGG CTA TGG ATT CAA C	TGG CGT CCC GCA TCA G
PPARα	GCA GAG GTC CGA TTC TTC CA	TGG GAT AGC CTT GGC AAA TT
ΡΡΑRδ	TCC ACA CCA GGC CCT TCT C	CCT TCA TCA TCC ACG ACA TTG A
UCP1	GAA ACG CCT GCC TCT TTG G	CAC GCC ACC CGT CAT CA
UCP2	TTG CCT CCC CCG TTG AT	TAC TGG CCC AAG GCA GAG TT
UCP3	CAC ATG GTG GAA GGA CAG GAT	TCT AGG GCG CTC GTA TGG A
adiponectin	AGG GCC AGG AGC TTT GGT	GCC ATC CAA CCT GCA CAA G
leptin	CTT CAT TCC CGG GCT TCA	GCC AGG GTC TGG TCC ATC TT
S17	CCG GGT CAT CAT CGA GAA GT	CGC TTG TTG GTG TGA AAG TCA

 Table 2-2
 Sequence of PCR primers

	Diet group						
	С	C20%	HF	HF20%			
Initial body weight (g)	85.0 ± 1.9	85.0 ± 3.0	85.0 ± 1.9	85.2 ± 2.7			
Final Body weight (g)	300.5 ± 4.0^{ab}	$286.6\pm4.2~^{\rm a}$	$316.5\pm6.8~^{\rm b}$	$305.1\pm7.5~^{ab}$			
Body weight gain (g)	215.4 ± 4.4^{ab}	201.6 ± 1.7 $^{\rm a}$	$231.3\pm5.4~^{\rm b}$	219.8 ± 6.2^{ab}			
Food intake (kcal/day/rat)	60.9	58.7	69.9	66.6			
Food efficiency *	0.0158	0.0175	0.0148	0.0168			

 Table 2-3
 Effects of ESG on the body weight change, food intake and food efficiency

Data of body weights are means \pm S.E. n = 8 (C and HF) or 7 (C20% and HF20%)

Food intake and efficiency are expressed as the group score.

The means in each row with different superscript letters are significantly different (p < 0.05).

* Food efficiency is calculated by following formula; [(Total body weight gain (g)) / (Total calorie intake (kcal)]

	8	, , , , ,	,					
	Diet group							
С		C20%	C20% HF					
		(% bod	y weight)					
Visceral fat								
mesenteric	1.48 ± 0.05 $^{\rm a}$	1.24 ± 0.04 $^{\rm a}$	$1.78\pm0.11~^{b}$	1.45 ± 0.07 $^{\rm a}$				
perinephric	2.01 ± 0.07 $^{\rm a}$	1.75 ± 0.12 $^{\rm a}$	2.70 ± 0.14 b	2.18 ± 0.11 a				
epididymal	1.55 ± 0.07 $^{\rm a}$	1.34 ± 0.12 a	2.06 ± 0.13 b	$1.76\pm0.14~^{ab}$				
Liver	6.97 ± 0.27 $^{\rm a}$	6.70 ± 0.11 $^{\rm a}$	5.94 ± 0.22 $^{\rm b}$	$6.45\pm0.07~^{ab}$				
Pancreas	0.13 ± 0.02	0.11 ± 0.02	0.11 ± 0.02	0.12 ± 0.02				
Cecum	1.04 ± 0.08 $^{\rm a}$	1.77 ± 0.11 $^{\rm b}$	0.78 ± 0.08 $^{\rm a}$	$1.46\pm0.10^{\ b}$				
Cecal content	0.73 ± 0.07 a	$1.32\pm0.08~^{\text{b}}$	0.54 ± 0.07 $^{\rm a}$	1.06 ± 0.10 $^{\rm b}$				

 Table 2-4
 Effects of ESG on the weights of visceral fat, liver, pancreas, cecum and cecal content

Data are means \pm S.E. n = 8 (C and HF) or 7 (C20% and HF20%)

The means in each row with different superscript letters are significantly different (p < 0.05).

	Diet group						
_	С	C20%	HF	HF20%			
Glucose (mg/dL)	171 ± 12	179 ± 12	172 ± 16	157 ± 15			
TG (mg/dL)	127 ± 15 $^{\rm a}$	115 ± 17 $^{\rm a}$	54 ± 6 b	61 ± 7 ^b			
Cholesterol (mg/dL)	87 ± 7	71 ± 7	51 ± 6	66 ± 5			
NEFA (meq/L)	$0.88\pm0.14~^{ab}$	$0.98\pm0.13~^{b}$	0.51 ± 0.04 a	0.52 ± 0.08 a			
TBA (mmol/L)	6.1 ± 1.0 ^a	6.2 ± 1.0 ^{ab}	5.6 ± 0.6 $^{\rm a}$	$9.8\pm1.0~^{\text{b}}$			
Adiponectin (ng/ml)	6.5 ± 0.47 $^{\rm a}$	5.6 ± 0.33 $^{\rm a}$	5.1 ± 0.53 $^{\rm a}$	3.0 ± 0.26 b			
Insulin (ng/ml)	3.2 ± 0.52	2.7 ± 0.70	1.8 ± 0.53	2.7 ± 0.29			

 Table 2-5
 Effects of ESG on blood glucose, TG, CHL, NEFA, BA, adiponectin, insulin levels

Data are means \pm S.E. n = 8 (C and HF) or 7 (C20% and HF20%)

The means in each row with different superscript letters are significantly different (p < 0.05).









D



Fig. 2-1 The evaluation of lipid accumulation induced by a high-fat diet by CT analysis.

Rats were fed the indicated diet for 3 weeks and were then scanned using an experimental animal X-ray CT system (Latheta LCT-100). Total fat (A), subcutaneous fat (B) and visceral fat (C) were calculated as the percentages (w/w) and expressed as means \pm S.E. [(n = 8 (C and HF) or 7 (C20% and HF20%)]. CT images of the typical patterns are shown (D). Different letters in the bar means significantly different from each other (p < 0.05).



Fig. 2-2 The amount of hepatic lipid storage.

Total lipid (A), TG (B), total cholesterol (C), and PL (D) were shown as means \pm S.E. [(n = 8 (C and HF) or 7 (C20% and HF20%)]. Different letters in bars show significant difference (p < 0.05).



Fig. 2-3 The amount of lipid excretion to the feces.

Total lipid (A), TG (B), total cholesterol (C), and PL (D) were shown as means \pm S.E. [(n = 8 (C and HF) or 7 (C20% and HF20%)]. Different letters in bars show significant difference (p < 0.05).







Fig. 2-4 Expression of mRNA related to lipid metabolism, in liver, muscle and adipose tissue

The expression levels of target mRNA were quantified by real-time RT-PCR with the primers indicated in Table 2-2 and shown as means \pm S.E. [(n = 8 (C and HF) or 7 (C20% and HF20%)]. Different letters in bars show significant difference (p < 0.05).

Chapter 3

Effects of ESG on lipid metabolism in diet induced obese mice

Introduction

The number of overweight (BMI \geq 25) has been more than 1.9 billion adults and it is triple since 1975, according to the fact sheet of World Health Organization (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight). It has been well studied that the excess accumulation of lipid in the adipose tissue rise the risk of some diseases, such as Alzheimer disease, type II diabetes, hypertension, ischemia heart diseases, certain cancers, and hyperlipidemia [77-83]. Recently, beneficial effects of many foods and food components such as catechins, green tea, black tea, wasabi leaf and almonds on the suppression of obesity have been reported [84-89].

As described in Chapter 1, ESG possess resistant property against digestive enzymes, and the glycemic index of it is estimated 79 from the result of oral glucose tolerance test in rats [13, 56]. It is suggested that the α -macrodextrin from ESG is utilized by *Bifidobacterium* and *Lactobacillus* to produce SCFAs in rat [56]. Thus, the author considered that supplementation of ESG could act as like a dietary fiber. There are many reports mentioned anti-obesity effects of dietary fiber [90, 91]. Actually, supplementation of ESG inhibited body fat mass accumulation and lipid accumulation in the liver in rat as shown in Chapter 2 [92], although only the limited information of ESG on lipid metabolism have been revealed.

The author investigated the effects of long-period supplementation of ESG on the lipid metabolism in obese mice induced by high-fat diet in this chapter. Rats were fed a normal or high-fat diet with or without ESG for 15 weeks. Then, the author measured the lipid accumulation in the adipose tissue, plasma lipids concentration, the lipids content in the liver, lipids excretion in the feces, body temperature, and NO production to assess the effects of ESG. After that, the effect of ESG on lipid absorption was evaluated in a single administration test.

Materials & Methods

Materials

ESG used in this study was synthesized by the method previously described [12]. All other chemicals, unless otherwise specified were of the analytical grade obtained from Wako Pure Chemical Industries. Animal diets were purchased from Research Diets, Inc. (New Brunswick, NJ).

Animal experiments

Before the start of animal experiments, the plans were approved by the Institutional Animal Care and Use Committee (Permission number: 21-07-02) in Kobe university. Experiments were conducted according to the Kobe University Animal Experimentation Regulations. C57BL/6NCr mice (male, 4 weeks) purchased from Japan SLC were housed in the same condition described in Chapter 2. The mice were randomly assigned to C-0%, C-10%, C-20%, HF-0%, HF-10%, and HF-20% group (n=5) after 1 week quarantine. ESG was added to the normal diet (No. D12450B:) and a high-fat diet (No. D12492) containing 60% kcal lard at the levels of 10% or 20% (w/w). The diet composition is shown in Table 3-1. ESG was replaced to starch and cellulose, because ESG contains approximately 17-22 % of dietary fiber [13]. The author measured the body weight and food intake once a week during the experiment. After 14-h fasting, mice were scarified and the interscapular brown adipose tissue, mesenteric, retroperitoneal, epididymal and subcutaneous white adipose tissues, liver, muscle, and cecum samples were collected at the end of the experiment. These tissues were weighted and washed each tissue with KCl solution (1.15%) after collection and stocked at -80° C.

Body temperature

The author measured the body temperature on the day before the end of the test

by the thermometer TD-300 (Shibaura Electronics Co., Ltd., Saitama, Japan) for 30 sec at 2.5 cm depth in the rectum.

Body composition

The body composition of mice was analyzed by animal X-ray CT system at 14th week as described in Chapter 2.

Blood lipids and NO

The concentrations of plasma TG, total cholesterol, NEFA, and glucose were measure as described in Chapter 2. Plasma NO level of each sample was measured after deproteinization by using the NO₂/NO₃ Assay Kit-FX (Fluorometric) purchased from DOJINDO LABORATORIES (Dojin Chemical, Tokyo, Japan). *The contents of hepatic and fecal lipid*

The freeze-dried liver and feces collected at the day before sacrifice were ground to powder, then lipid contents were measured as described in Chapter 2. *Effect of ESG on the lipid accumulation*

To investigate the effect of ESG on lipid absorption, 36 mice were purchased from Japan SLC and divided into control and ESG group. After 16-h starvation, mice were orally administered with 5 ml/kg B.W. of corn oil emulsion (3ml of corn oil, 3ml of water, 50 mg of lecithin and 50 mg of cholic) simultaneously with 5 ml/kg B.W. of water or 5% ESG solution. The author collected blood samples from tail vein before, 1, 2, 3, 4 and 6 hours after administration, and the concentration of plasma TG was measured by the kit as described above.

Statistical analysis

Data are shown as means \pm SE in the table and figure. The statistical analysis was conducted by one-way ANOVA followed by Tukey-Kramer test in the 15-weeks supplementation experiment. In the lipid administration test, statistical significance was analyzed by student's *t*-test. When *p* values were < 0.05 the

differences were considered as significant.

Results

Body weight, body composition and adipose tissue weight

The results from CT analysis at 14 weeks revealed significant increase in total fat mass in the HF-0% group compared with the C-0% group. Total volume of adipose tissue in the HF-0% group was significantly higher than that in the HF-20% group (Fig. 3-1). The food intake was not affected by ESG in all groups. The body weight was significantly higher in HF-0% group than the C-0% group after 15-week (Table 3-2). ESG significantly suppressed body weight gain in the HF-20% group compared to the HF-0% group. The relative weights of white adipose tissues (retroperitoneal mesenteric, epididymal and subcutaneous) were increased by high-fat diet, while those were significantly decreased by 20% ESG (Table 3-2). Neither high-fat diet nor ESG affected the relative brown adipose tissue weight.

The blood levels of lipids and NO

In the HF-0% group, the concentration of plasma glucose significantly increased compared to the C-0% group (Table 3-3), and ESG tended to but not significantly suppressed that increase. High-fat diet significantly increased the total cholesterol level, and 20% ESG supplementation suppressed this increase. Neither high-fat diet nor ESG changed the plasma levels of TG and NEFA. Since thermogenesis in the brown adipose tissue is increased by NO [93], the NO level in plasma was measured. The concentration of plasma NO was significantly enhanced by ESG in the C-20% group compared to C-0% group, while the NO level in the HF group was not increased by ESG though an increasing tendency (Table 3-3).

Body temperature

Since the possible effect of ESG on energy metabolism was suggested, the

author measured body temperature. The body temperature in the C-0%, C-10%, C-20%, HF-0%, HF-10%, and HF-20% groups were 37.4 ± 0.2 , 37.8 ± 0.1 , 38.3 ± 0.2 , 37.6 ± 0.1 , 37.5 ± 0.1 , and $37.4 \pm 0.2^{\circ}$ C, respectively. The body temperatures in the C-20% group was markedly higher than that of the C-0% group, although there were no significant difference between the C-0% and the HF-0% groups and among the high-fat diet groups.

Hepatic lipids

Neither high-fat diet nor ESG affected the relative and net liver weight (Table 3-2 and 3-4). In the HF-0% group, the total lipid level in the liver significantly increased compared with the C-0% group and ESG suppressed this increase in a dose-dependent manner. The increase in hepatic TG, phospholipid and cholesterol induced by high-fat diet were also significantly suppressed by 20% ESG.

Fecal lipid and cecum weight

Supplementation with 20% ESG in both control and high-fat diets significantly increased the cecum weights (Fig. 3-2A) and ESG also increased cecal content (Fig. 3-2B). The excretion amount of fecal lipid was significantly increased by 49% and 56% in the HF-10% and HF-20% groups, respectively, compared with the HF-0% group (Fig. 3-2C), while there was no significant change among the control groups.

Oral lipid administration test

When the single oral administration test was performed, ESG significantly reduced the concentration of plasma TG at 2 hours after the administration of lipid (Fig. 3-3). This result suggested that ESG suppress the absorption of lipid in mice.

Discussion

In this chapter, the author evaluated the effects of long-term supplementation of

ESG on the lipid accumulation, excretion and metabolism in both normal and highfat diet-induced obese mice. In HF groups, ESG increased the excretion of lipids into the feces, but not in the control diet fed groups (Fig.3-2). These data suggested ESG suppressive effect of ESG on lipid absorption, in fact, the rise of plasma TG was significantly suppressed by ESG after oral administration of corn oil emulsion (Fig. 3-3). Thus, the inhibition of lipid absorption is considered to be the one of the mechanism of the effect of ESG.

There are many reports indicate effect of food ingredients on the lipid absorption. Catechins and flavonoids inhibit the activity of lipase and lipid absorption [94, 95]. Resistant dextrin is reported to suppress the postprandial increase of plasma TG [96]. In these reports, the mechanism of inhibitory effect is considered inhibition of the decomposition of micelle and stabilization of micellar structure. ESG could work as a water soluble dietary fiber, but it did not affect the micellar stability or lipase activity (data not shown). Farther study are needed to clarify the exact mechanism of the effect of ESG on lipid absorption.

In this chapter, lipid accumulation in the liver and adipose tissues induced by high-fat diet were significantly suppressed by supplementation of 20% ESG but not by 10% ESG, although the amounts of the lipid excretion to the feces in the HF-10% and HF-20% groups were same level (Figs.3-1 and 3-2). These results indicate that there are other mechanisms of the effect of ESG on the lipid accumulation.

Supplementation of ESG significantly increased the body temperature and the concentration of plasma NO in the C-20% group (Table3-3). Since it has been reported that ESG treatment in increases the NO production of macrophages [15], it is possible that the plasma NO level was increased by ESG through enhancement of the production of NO in the small intestine by macrophages. However, the author could not conclude the effect of ESG on thermogenesis, because ESG did neither

affect body temperature nor NO level at all in HF groups.

There could be other possibility of the relationship of gut bacteria. ESG and certain glycogens have resistant property to the digestion of α -amylase [13]. The results in Chapter 1 showed that ESG is converted to SCFAs by intestinal microbe such as *Lactobacillus* and *Bifidobacterium* [56]. The increase in the amount of cecal content indicated the prebiotic effect of ESG in this chapter as well. There have been many reports suggested that prebiotics possess the preventive effect on obesity through the changes of intestinal microbiota [Review in 97]. Thus, the author considered, at least in part, the prebiotics effect could relate to the anti-obese effect of ESG.

Taken together my findings, ESG revealed the inhibitory effect of on the lipid accumulation in the liver and adipose tissues, and the plasma cholesterol level in diet-induced obese model mice, while anti-obesity effects of ESG on humans have not been studied. Thus, future studies to evaluate the effect of ESG on anti-obesity in a human clinical study and understanding the detailed mechanism in further animal studies are necessary to establish the evidence for ESG to use for human health.

Table 3-1 Compositions of the control and high-fat diets

Group						
	Control				HF	
0%	10%	20%	0%	10%	20%	
19.0	19.0	19.0	25.8	25.8	25.8	
0.28	0.28	0.28	0.39	0.39	0.39	
29.9	21.6	13.3	0	0	0	
3.3	3.3	3.3	16.2	8.3	0	
33.2	33.2	33.2	8.9	8.9	8.9	
0	10	20	0	10	20	
4.7	3.0	1.3	6.5	4.3	2.6	
2.4	2.4	2.4	3.2	3.2	3.2	
1.9	1.9	1.9	31.7	31.7	31.7	
0.95	0.95	0.95	1.3	1.3	1.3	
1.2	1.2	1.2	1.7	1.7	1.7	
0.52	0.52	0.52	0.71	0.71	0.71	
1.6	1.6	1.6	2.1	2.1	2.1	
0.95	0.95	0.95	1.3	1.3	1.3	
0.19	0.19	0.19	0.26	0.26	0.26	
abolizable energy	r (%)					
20	20	20	20	20	20	
70	70	70	20	20	20	
10	10	10	60	60	60	
	0% 19.0 0.28 29.9 3.3 33.2 0 4.7 2.4 1.9 0.95 1.2 0.52 1.6 0.95 0.19 abolizable energy 20 70 10	$\begin{tabular}{ c c c c } \hline Control \\ \hline 0\% & 10\% \\ \hline 19.0 & 19.0 \\ 0.28 & 0.28 \\ 29.9 & 21.6 \\ 3.3 & 3.3 \\ 33.2 & 33.2 \\ 0 & 10 \\ 4.7 & 3.0 \\ 2.4 & 2.4 \\ 1.9 & 1.9 \\ 0.95 & 0.95 \\ 1.2 & 1.2 \\ 0.52 & 0.52 \\ 1.6 & 1.6 \\ 0.95 & 0.95 \\ 0.19 & 0.19 \\ \hline abolizable energy (%) \\ \hline 20 & 20 \\ 70 & 70 \\ 10 & 10 \\ \hline energy (%) \\ \hline \end{tabular}$	$\begin{tabular}{ c c c c } \hline \hline Control \\ \hline \hline 0\% & 10\% & 20\% \\ \hline 19.0 & 19.0 & 19.0 \\ 0.28 & 0.28 & 0.28 \\ 29.9 & 21.6 & 13.3 \\ 3.3 & 3.3 & 3.3 \\ 33.2 & 33.2 & 33.2 \\ 0 & 10 & 20 \\ 4.7 & 3.0 & 1.3 \\ 2.4 & 2.4 & 2.4 \\ 1.9 & 1.9 & 1.9 \\ 0.95 & 0.95 & 0.95 \\ 1.2 & 1.2 & 1.2 \\ 0.52 & 0.52 & 0.52 \\ 1.6 & 1.6 & 1.6 \\ 0.95 & 0.95 & 0.95 \\ 0.19 & 0.19 & 0.19 \\ \hline abolizable energy (\%) \\ \hline 20 & 20 & 20 \\ 70 & 70 & 70 \\ 10 & 10 & 10 \\ \hline \end{tabular}$	$\begin{tabular}{ c c c c c } \hline \hline Control & \hline \hline Control & 0\% & 10\% & 20\% & 0\% \\ \hline \hline 0\% & 10\% & 20\% & 0\% & \hline \\ \hline 19.0 & 19.0 & 19.0 & 25.8 & 0.39 & 0.28 & 0.28 & 0.39 & 0.28 & 0.39 & 0.33 & 3.3 & 3.3 & 16.2 & 33.2 & 32.2 & 1.9 & 31.7 & 0.95 & 0.95 & 0.95 & 1.3 & 0.19 & 0.19 & 0.19 & 0.26 & 0.05 & 0.95 $	$\begin{tabular}{ c c c c c c } \hline Control & \hline \hline Control & \hline \hline 0\% & 10\% & 20\% & 0\% & 10\% \\ \hline \hline 0\% & 10\% & 20\% & 0\% & 10\% & 10\% & 19.0 & 25.8 & 25.8 & 0.28 & 0.28 & 0.39 & 0.30 & 0.30 & 0.30 & 0.30 & 0.30 & 0.30 & 0.30 & 0.30 &$	

HF, high fat; ESG, enzymatically synthesized glycogen

	-	_		Group			
		Control			HF		
	0%	10%	20%	0%	10%	20%	
Body weight (g)	26.4 ± 0.6^{a}	25.1 ± 0.3^{a}	26.1 ± 1.5^{a}	38.2 ± 1.7^{b}	39.1 ± 1.3^{b}	34.4 ± 1.7^{b}	
Tissue weight (g/100 g body	weight)						
Total white adipose	9.22 ± 0.49^{a}	$8.19 \ \pm 0.66^{a}$	5.71 ± 0.89^a	24.78 ± 1.08^{b}	$24.12 \ \pm 1.09^{b}$	$17.40\pm1.54^{\rm c}$	
Epididymal	2.26 ± 0.11^{a}	1.98 ± 0.21^{a}	$1.45 \ \pm 0.18^{a}$	5.64 ± 0.17^{b}	5.48 ± 0.22^{b}	4.57 ± 0.47^{b}	
Mesenteric	1.03 ± 0.07^{a}	0.86 ± 0.06^{a}	$0.58\pm0.11^{\rm a}$	$2.62 \pm 0.41^{\circ}$	$2.26\ \pm 0.17^{bc}$	1.48 ± 0.21^{ab}	
Retroperitoneal	1.10 ± 0.07^{a}	$0.81 \ \pm 0.11^{a}$	$0.50\ \pm 0.10^a$	2.93 ± 0.16^{b}	$2.77\ \pm 0.07^b$	2.37 ± 0.30^{b}	
Subcutaneous	4.84 ± 0.39^{a}	$4.54\ \pm 0.31^{ab}$	3.18 ± 0.51^{a}	13.59 ± 0.84^{b}	13.61 ± 0.81^{b}	$8.98 \pm 0.69^{\circ}$	
Brown adipose	$0.46\pm0.06^{\rm a}$	0.36 ± 0.03^{a}	$0.36 \ \pm 0.05^{a}$	$0.51 \ \pm 0.09^{a}$	$0.50 \ \pm 0.04^{a}$	0.43 ± 0.04^{a}	
Liver	3.28 ± 0.28^{a}	3.61 ± 0.27^{a}	3.50 ± 0.09^{a}	2.49 ± 0.23^{a}	2.74 ± 0.07^{a}	$2.76 \ \pm 0.18^{a}$	

Table 3-2 Effects of ESG on body and tissue weight of mice fed control and high-fat diets for 15 weeks

HF, high fat

Mice were fed the control or high-fat diet containing 0%, 10%, and 20% ESG for 15 weeks. At the end of experiment, body weight and tissue weights were measured after 14 h fasting.

Values are the mean \pm S.E (n = 4, 5). Values without a common letter in a row differ significantly (p < 0.05)

by the Tukey-Kramer multiple comparison test.

Table 3-3 Effects of ESG on blood parameters

	Group						
		Control		HF			
	0%	10%	20%	0%	10%	20%	
Glucose (mg/dl)	168 ± 5^{ac}	171 ± 5^{ab}	148 ± 14^{a}	$216~\pm~10^{\rm b}$	206 ± 16^{bc}	188 ± 11^{ab}	
Total cholesterol (mg/dl)	132 ± 7^{ab}	121 ± 13^{ab}	100 ± 7^{a}	$187 \pm 11^{\circ}$	152 ± 8^{bc}	106 ± 7^{a}	
NEFA (meq/l)	0.65 ± 0.07^{a}	0.66 ± 0.15^{a}	0.67 ± 0.08^{a}	0.73 ± 0.06^{a}	0.76 ± 0.06^{a}	0.66 ± 0.06^{a}	
Triglyceride (mg/dl)	42 ± 3^{a}	43 ± 8^{a}	45 ± 6^{a}	60 ± 8^{a}	55 ± 3^{a}	55 ± 6^{a}	
NO ²⁻ +NO ³⁻ (µmol/l)	24 ± 1^{a}	43 ± 5^{a}	64 ± 10^{b}	24 ± 3^{a}	25 ± 2^{a}	35 ± 6^{a}	

HF, high fat; NEFA, non-esterified fatty acid; NO, nitric oxide

Mice were fed the control or high-fat diet containing 0%, 10%, and 20% ESG for 15 weeks. At the end of experiment, blood parameters were measured after 14 h fasting.

Values are the mean \pm S.E (n = 4, 5). Values without a common letter in a row differ significantly (p < 0.05)

by the Tukey-Kramer multiple comparison test.

Table 3-4 Effects of ESG on liver weight and lipid levels

		Group							
		Control			HF				
	0%	10%	20%	0%	10%	20%			
Liver weight (g)	0.87 ± 0.10^{a}	0.90 ± 0.06^{a}	0.91 ± 0.06^{a}	1.15 ± 0.13^{a}	1.07 ± 0.04^{a}	0.94 ± 0.06^{a}			
Total lipids (mg/g liver)	86.5±4.1 ^a	$74.0~\pm~2.5^a$	78.1 ± 8.2^{a}	190.3 ± 30.8^{b}	163.0 ± 12.7^{bc}	104.6 ± 7.8^{ac}			
Phospholipids (mg/g liver)	19.4 ± 0.9^{a}	16.9 ± 0.5^{a}	17.7 ± 1.4^{a}	$57.7~\pm~5.2^{b}$	52.9 ± 3.4^{bc}	$40.4 \pm 4.7^{\circ}$			
Triglycerides (mg/g liver)	36.2 ± 3.0^{ab}	$33.9~\pm~2.4^a$	32.9 ± 3.3^{a}	$90.5~\pm~8.6^{\circ}$	$82.0 \pm 4.9^{\circ}$	$58.4\ \pm 5.8^b$			
Total cholesterol (mg/g liver)	6.3 ± 0.4^{a}	4.6 ± 0.5^a	$4.5~\pm~0.8^a$	$12.7~\pm~1.8^{b}$	$11.9~\pm~0.8^{b}$	$5.3\ \pm 0.8^a$			

HF, high fat

Mice were fed the control or high-fat diet containing 0%, 10%, and 20% ESG for 15 weeks.

Values are the mean \pm S.E (n = 4, 5). Values without a common letter in a row differ significantly (P < 0.05) by the Tukey-Kramer multiple comparison test



Fig. 3-1 CT analysis of body fat percentage

The body fat was analyzed by X-ray CT as described in Materials & Method at 14th week. Different letters in bars show significant difference (p < 0.05, Tukey-Kramer multiple comparison test).





The cecum (A) cecal contents (B) were weighted after 14-h starvation at the end of the experiment. The feces were collected and lipids contents were measured as described in Materials & Methods at 3 days before the end of the experiment (C). Different letters in bars show significant difference (p < 0.05, Tukey-Kramer multiple comparison test).



Fig. 3-3 Effects of ESG on lipid absorption in mice.

Mice were starved for 16 hours before the administration of corn oil emulsion simultaneous with water or ESG solution. Blood was collected from tail vein at before and 1, 2, 3, 4 and 6 hours after the administration. The concentration of plasma TG was measured by commercial kit. Asterisk shows significant difference (p < 0.05, student's *t*-test).

Chapter 4

Effects of oral ESG supplementation on cognitive function: a doubleblind, randomized, placebo-controlled study

Introduction

In Chapter1 to Chapter3, the author demonstrated dietary fiber like, prebiotics and anti-obese effects of ESG. My collaborators reported that the immunomodulatory effects of ESG in detail [15, 16, 17, 18 and 25]. These results suggest that ESG not only has a role in maintaining the blood glucose level, but also has health beneficial effects. The evidence from human clinical study is necessary to use as functional ingredient of health-promoting food, but ESG have not been revealed health benefit in human study. The author considered to conduct human clinical study to proof the effects on lipid absorbance or intestinal microbiota. However in the market there are so many functional food claimed to reduce lipid absorption and improve intestinal microbiota. As described in General Introduction, in recent years, it has been reported that strengthening the immunity and improving the intestinal environment enhance the cognitive function in the brain [19-21], and prebiotic and indigestive polysaccharide have been reported to possess beneficial effects on brain function [21-24].

It is well known that cognitive function gradually decreases with aging, while it declines dramatically in patients with dementia such as Alzheimer's disease [98]. In addition, cognitive performance is decreased by alcohol intake, acute and chronic fatigue, and stress [99, 100]. Because cognitive deficits in areas such as attention, memory, and reaction time reduce the quality of life, improvement of cognitive performance is a target of much research. It has been reported that astaxanthin and flavonoids can improve age-related cognitive decline. For example, administration of astaxanthin for 12 weeks significantly improved the cognitive performance of elderly subjects, who complained of age-related forgetfulness [101]. The cognitive function of the subject was evaluated by the CogHealth test, which is designed to evaluate cognitive performance by playing card

games on a personal computer and can detect slight changes in healthy or mildly impaired subjects at an early date [101, 102]. It was also reported that a flavonoid-rich extract of *Pinus radiata* bark improves cognitive function in terms of immediate recognition and spatial working memory [103]. These reports suggest that oral administration of certain food components may enhance cognitive function.

In this chapter, the author examined whether the cognitive performance of healthy subjects was enhanced by oral administration of ESG using a double-blind, randomized, placebo-controlled, cross-over study design. The author found that oral administration of ESG for 2-4 weeks significantly potentiated the cognitive performance of healthy volunteers, including the reaction time in the "choice reaction task" and accuracy in the "one card learning task". To the best of my knowledge, this is the first report about the effect of orally administered glycogen on brain function in humans. My findings suggest that glycogen can potentially be used as a food component that improves cognitive function.

Materials & Methods

Preparation of ESG

ESG was prepared as described Chapter 1 [12]. In the present study, ESG was administered through a beverage. The compositions of the ESG beverage and placebo beverage are shown in Table 4-1. Fifty milliliters of the ESG beverage contained 5.0 g of ESG, 15 mg of citric acid, and 3.0 mg of sucralose, while the placebo beverage contained dextrin (α -1,4/1,6 glucans). For blinding, all beverages were sealed in identical aluminum bottles and sterilized. It was impossible to discriminate between the ESG and placebo beverages in terms of taste and appearance.

Subjects

Forty healthy adults (25 men and 15 women) aged between 25 and 58 years participated in this study. All subjects were in full-time employment and worked approximately 7.5 h/day on 5 days/week. Since 2 participants dropped out for personal reasons, data on the remaining 38 subjects were used for analysis. The mean \pm SD age, height, weight, and body mass index of these 38 subjects (23 men and 15 women) were 41.8 \pm 8.03 years (45.0 \pm 7.40 years for men and 36.9 \pm 6.50 years for women), 164.0 \pm 8.58 cm (169.4 \pm 6.05 cm and 155.9 \pm 4.39 cm), 61.0 \pm 11.2 kg (66.0 \pm 8.33 kg and 53.5 \pm 11.1 kg), and 22.6 \pm 3.39 kg/m² (23.0 \pm 2.97 kg/m² and 22.0 \pm 3.96 kg/m²), respectively (Table 4-2).

This study was conducted from November 2013 to February 2014. The study protocol and the informed consent document conformed to the guidelines of the Declaration of Helsinki, and were approved by the ethics committee of Ezaki Glico Co., Ltd (Registration Number: 2013-7). All participants provided written informed consent prior to participation.

Study design

This was a randomized, double-blind, placebo-controlled, cross-over study (Fig. 4-1). The primary endpoint was the effect of orally administered ESG on cognitive function, which was assessed by using the CogHealth test battery. Thirty-eight subjects were randomly divided into two groups (group A and B) by the person responsible for grouping. All of the subjects, study administrators, and data processors (except for the person responsible for grouping) were completely blind to the assignment of treatment until the end of the trial. During the first experimental period, the group A ingested the ESG beverage once daily between 13:00 and 18:00 for 4 weeks, while the group B ingested the placebo beverage. After a washout period more than 4 weeks, the beverages were reversed in the second 4-week experimental period. In each experimental period, cognitive testing and saliva sampling were carried out three times, i.e., before treatment (baseline) and after 2 weeks and 4 weeks of ingestion. On each day of testing, subjects ate Japanese noodles made from flour (460 kcal) at 12:00 - 12:30 as a lunch, after that other than water was prohibited to intake until 16:00. Subjects entered a test room at 16:00 and performed the cognitive test once in the training mode for about 5 min. Then, they rested for 20 min while sitting on the chair. A saliva sample was collected with a Salivette (Sartrious Co., Getting, Germany) at 16:25, and cognitive testing was started at 16:30. *Cognitive testing*

Cognitive performance was assessed by using the CogHealth test, which consists of a battery of four tasks: "simple reaction task", "choice reaction task", "working memory task" (short-term working memory), and "one card learning task" (delayed recall).

For the "simple reaction task", a card is presented face down in the center of the computer screen. When this card turns face up, participants are required to press the "k" key as quickly as possible. There are 35 trials per task, and the task is repeated again after the one card learning task.

The "choice reaction task" is similar to the "simple reaction task". Participants are required to indicate the color of the card suit (black or red) by pressing the "d" key or the "k" key depending on the color. This task is continued until the participant has made 30 correct responses or until the maximum time is reached.

The "working memory task" assesses short-term memory. A single card is presented in the center of the screen and it changes every 2 sec. Each time the card changes, the participant must indicate whether or not the new card is the same as the one just before by pressing either the "d" key or the "k" key. When the response is correct, the card is moved to a single pack. This task is continued until the participant has made 30 correct responses or until the maximum time is reached.

The "one card learning task" is performed to assess delayed recall. A single card is presented in the center of the screen and it changes every 2 sec. Each time the card changes, the participant must indicate whether or not the new card has already been presented in this task by pressing either the "d" key or the "k" key. When the response is correct, the card is moved to a single pack. This task is continues until the participant has made 42 correct responses or until the maximum time is reached.

To minimize the learning effect, all subjects trained in performing the CogHealth battery for one week before the trial.

Assessment of fatigue

The severity of fatigue experienced by the subjects was evaluated by using a simple horizontal visual analog scale (VAS). Subjects were instructed to make a mark on the fatigue VAS, which was a straight horizontal line 100 mm in length, according to the intensity of their fatigue. Results were expressed in millimeters from zero (no fatigue: the left end of the line) to 100 (severe fatigue: the right end of the line).

Collection and analysis of saliva

Before ingestion of ESG (baseline) and after 2 and 4 weeks of ESG intake, saliva was collected from all subjects. A cotton Salivette (Sartrious Co.) was placed in the oral cavity and chewed for 3 min, after which a saliva sample was obtained by centrifugation of the retrieved Salivette. All saliva samples were stored at -80°C until analysis.

Levels of fatigue-related factors in saliva samples were measured by using following assay kits: Secretory IgA (sIgA) and cortisol concentrations were determined
with the sIgA Salivary EIA Kit and Cortisol Salivary EIA Kit (Salimetrics Co.), while α amylase activity was measured with an assay from Oriental Yeast Co., Ltd. (Tokyo, Japan) *Statistical analysis*

All analyses were performed using SPSS 16.0J software for Windows (SPSS Japan Inc., Tokyo, Japan). The significance of differences between the ESG group and the placebo group was determined with the paired *t*-test. Differences between baseline parameters and those obtained at later times during the study were analyzed by using Dunnett's multiple comparison test. In all analyses, p < 0.05 was considered significant.

Results

Disposition of the subjects

The author recruited 40 subjects and 38 of them completed this trial. The other two subjects dropped out for personal reasons unrelated to ingestion of the ESG and placebo beverages.

Effect of ESG on cognitive performance

Data on cognitive performance (CogHealth battery) are shown in Table 4-3 as relative values compared with the baseline results. Reaction times in the "simple reaction task" and "working memory task" were significantly shorter after the intake of ESG beverage for 4 weeks compared with baseline, while no such effect was observed with the placebo beverage. The reaction time in the "choice reaction task" and accuracy in the "one card learning task" were significantly improved after 2 and 4 weeks of ESG beverage intake, compared with the results for the placebo beverage. These findings suggested that the intake of ESG could improve several aspects of cognitive performance such as visual discrimination and memory.

Effect of ESG on fatigue and fatigue-related factors in saliva

Table 4-4 shows the results obtained by measuring several fatigue-related factors in saliva and the VAS fatigue scores. Salivary levels of α -amylase activity, cortisol, and sIgA showed no significant differences between the results of ESG and placebo beverages. Similarly, there was no significant difference of the VAS fatigue score between the ESG and placebo beverages. Taken together, these results suggested that the intake of ESG did not affect fatigue during daily life.

Discussion

This randomized cross-over study was performed to investigate whether ESG enhanced cognitive performance, revealing that cognitive parameters assessed by the CogHealth battery such as visual discrimination and memory were significantly improved by intake of ESG compared with placebo (Table 4-3). Although both ESG and the placebo used in this study (dextrin) are $(1\rightarrow 4)(1\rightarrow 6)$ -linked α -D-glucans, ESG contains far more α -1,6 branches than dextrin and is a high-density spherical molecule [93]. These features of ESG may result in minimal penetration of digestive enzymes such as α -amylase and α -glucosidase, so that the enzymes gradually digest ESG molecules from the periphery. Since enzymatic degradation of ESG is much slower than for dextrin, ESG may reach the distal small intestine [13, 18, 92]. It is possible that this feature of ESG is important for exerting its functions on digestive organs such as the small intestine after oral administration. Since ESG is a form of glycogen produced from starch, it has very low toxicity and the oral LD₅₀ is > 2000 mg/kg/day [14], suggesting that it can potentially be used as a new food ingredient for improvement of cognitive performance. It is known that cognitive performance is impaired by alcohol intake and by fatigue related to sleep disruption [99, 100]. Falleti *et al.* reported that the blood alcohol concentration and sleep deprivation were closely related to cognitive function, and that similar impairment of cognitive performance was caused by a blood alcohol concentration of 0.05% or sustained wakefulness for 24 h [99]. These findings suggest that controlling fatigue could induce the improvement of cognitive performance. To investigate whether improvement of fatigue was relevant to the cognitive effect of ESG, the author examined salivary levels of sIgA, cortisol, and α -amylase, as well as the VAS fatigue score, before the start of ESG intake and after ESG ingestion for 2 and 4 weeks. However, the author found no significant differences of the salivary fatigue-related factors between subjects taking ESG and placebo drinks (Table 4-4). VAS scores also showed no significant difference between ESG and placebo drinks. These findings indicated that the mechanism by which ESG improves cognitive performance is unrelated to fatigue.

A possible mechanism underlying the effects of ESG may be involved in the activation of the immune system. It has been reported that deterioration of cognitive function is accelerated by age-related immunodeficiency, with the learning performance of mice showing significant impairment at 10 months after resection of the thymus [104]. Furthermore, Lal *et al.* reported that immune senescence can induce deterioration of cognitive function [105]. They irradiated young mice to inactivate the immune system and then injected spleen and bone marrow cells obtained from aged mice with impaired learning or cells from normal young mice. After injection of the immune cells from aged mice, the young mice showed similar learning deficits to aged animals, while young mice receiving the immune cells harvested from other young mice exhibited normal learning.

These findings suggest that a healthy immune system is involved in the maintenance of normal cognitive function. The author and corroborator's previous studies have shown that oral ESG ingestion activates the immune system [18]. For example, the collaborators found that oral administration of ESG to mice activated CD11b⁺CD11c⁺ cells, such as macrophages and dendritic cells in Peyer's patche, which is in the small intestinal mucosa with an important role in the immune response. In addition, splenic natural killer cells were activated by ESG treatment, while splenic sympathetic nervous activity was suppressed. Moreover, ingestion of ESG suppressed tumor proliferation in mice and prolonged survival [18]. Thus, my findings suggest that oral intake of ESG induces systemic immune activation. Such activation of the immune system by ESG may be related to improvement of cognitive performance in this chapter. Investigations to clarify the mechanism by which ESG alters cognitive function are currently underway in an animal model.

	Placebo beverage	ESG beverage
ESG (mg)	0	5000
Maltodextrin (mg)	5000	0
Scralose (mg)	3.0	3.0
Citric acid (mg)	15	15
Total volume (mL)	50	50
Energy (kcal)	20	20
Protein (g)	< 0.05	< 0.05
Lipid (g)	< 0.05	< 0.05
Carbohydrate (g)	5.0	5.0
Sodium (mg)	0	0

Table 4-1 Composition of the placebo and ESG beverages

*Each beverage was ingested once daily (50 ml).

	Subjects					
	All (n=38)		Men (n=23)		Women (n=15)	
Characteristic	Mean	SD	Mean	SD	Mean	SD
Age (y)	41.8	8.03	45.0	7.40	36.9	6.50
Height (cm)	164.0	8.58	169.4	6.05	155.9	4.39
Weight (kg)	61.1	11.2	66.0	8.33	53.5	11.1
BMI (kg/m ²)	22.6	3.39	23.0	2.97	22.0	3.96

Table 4-2 Baseline characteristics of the subjects

Two dropouts were excluded from this analysis.

	Baseline	2 weeks		4 weeks		
	Mean±SD	Mean±SD	<i>p value</i> (vs Placebo)	Mean±SD	<i>p value</i> (vs Placebo)	
Simple reaction task:	reaction time					
Placebo	100.0 ± 15.35	99.10 ± 12.91		94.61 ± 21.68		
ESG	100.0 ± 17.91	98.16 ± 13.42	<i>N</i> . S.	94.10 ± 20.16	* <i>N. S.</i>	
Choice reaction task:	reaction time					
Placebo	100.0 ± 14.93	101.6 ± 14.14		99.58 ± 16.21		
ESG	100.0 ± 18.08	97.81 ± 13.21	p < 0.05	101.4 ± 21.47	\square N. S.	
Working memory task	c: reaction time					
Placebo	100.0 ± 18.25	97.58 ± 15.83		96.50 ± 17.96		
ESG	100.0 ± 17.26	95.63 ± 14.84	* <i>N. S.</i>	94.76 ± 17.87	* N. S.	
One card learning tas	k: reaction time					
Placebo	100.0 ± 39.58	97.36 ± 26.24		95.07 ± 24.54		
ESG	100.0 ± 21.98	98.27 ± 26.41	N. S.	97.36 ± 23.76	\square N. S.	
Working memory task	<pre>c: accuracy</pre>					
Placebo	100.0 ± 5.624	100.9 ± 4.513	–	99.40 ± 5.822		
ESG	100.0 ± 4.176	99.51 ± 3.543	N. S.	99.84 ± 3.112	\square N. S.	
One card learning task	k: accuracy					
Placebo	100.0 ± 14.74	99.58 ± 13.41		99.42 ± 13.90	$\int n < 0.05$	
ESG	100.0 ± 14.66	101.8 ± 13.48	IV. 3.	104.5 ± 15.33	p < 0.05	

Table 4-3 Relative mean reaction times and accuracy in the CogHealth test battery at baseline and after 2 and 4 weeks of ESG intake

Two dropouts were excluded from data analysis. *: p < 0.05 vs. baseline. *N.S.*: not significant vs. placebo group. Data are relative value compared to baseline. Differences between the ESG group and placebo group were determined with the paired *t*-test. Multiple comparisons between 2 or 4 weeks and baseline were performed using Dunnett's test.

	Baseline	2 weeks		4 weeks	
	Mean±SD	Mean±SD	<i>p value</i> (vs Placebo)	Mean±SD	<i>p value</i> (vs Placebo)
Fatigue VAS (mm)					
Placebo	52.46 ± 19.65	51.62 ± 18.84		52.45 ± 15.67	
ESG	53.83 ± 16.84	50.51 ± 18.51	\square N. S.	52.99 ± 18.05	\square N. S.
sIgA (mg/ml)					
Placebo	23.03 ± 14.38	25.10 ± 14.19		23.02 ± 12.11	
ESG	23.59 ± 17.69	25.46 ± 16.33	N. S.	20.47 ± 17.44	N. S.
a-Amylase activity	(IU/mL)				
Placebo	200.7 ± 134.9	213.1 ± 140.4		239.0 ± 156.4	*]
ESG	204.5 ± 134.5	225.4 ± 151.2	N. S.	244.1 ± 179.2	* <i>N</i> . S.
Cortisol (ng/dL)					
Placebo	94.07 ± 51.95	134.8 ± 105.0	**]	123.8 ± 70.43	**]
ESG	89.91 ± 64.21	115.6 ± 50.29	** N. S.	130.4 ± 64.93	** <i>I</i> V. <i>S</i> .

Table 4-4 Mean fatigue VAS score and salivary parameters at baseline and after 2 and 4 weeks of ESG ingestion

Two dropouts were excluded from data analysis. *: p<0.05 vs baseline. **: p<0.01 vs baseline. N.S.: not significant vs placebo group. Differences between the ESG group and placebo group were determined with the paired *t*-test. Multiple comparisons between 2 or 4 weeks and baseline were performed using Dunnett's test.



Fig. 4-1 Flow diagram of the study

General discussion

In this thesis, the author used ESG to examine the metabolic fate in rats, anti-obesity effect in rats and mice, and the effect on cognitive function in humans to obtain new knowledge about the health promoting function of ESG. First, in Chapter 1, metabolic fate of ESG was examined when it is ingested orally in rat. In Chapters 2 and 3, the anti-obesity effect, which is expected from the dietary fiber-like effect of ESG revealed in Chapter 1, was assessed in the diet-induced obese model rats and mice. In Chapter 4, the author demonstrated in clinical trials that oral ingestion of ESG has a positive effect on cognitive function in healthy humans.

It was my best knowledge that glycogen is considered to be completely degraded by digestive enzymes because it is recognized as energy source. However, the author and collaborators previously suggested that ESG and some naturally derived glycogen are resistant to digestive enzymes in *in vitro* studies [13]. In Chapter 1, as the result of oral administration test in rats, it was confirmed that the glycemic index of ESG was 79 and the amount of glucan content in cecum was increased by ESG supplementation, thus the author considered resistance property of ESG to digestive enzymes was also shown *in vivo*. This finding is meaningful knowledge which lead to understanding about health benefit of ESG. On the other hand, digestion tolerance *in vivo* was not confirmed in natural glycogen used in this thesis. This difference was thought to be due to the difference in fine internal structure. As natural glycogens have a slight difference in internal structure depending on the difference in origin, it is possible that some natural glycogens could have resistance to digestive enzymes *in vivo* like ESG. Further research is needed to complete understanding of the digestive tolerance of glycogens.

It has been clarified that ESG has a digestion-resistant fraction, and it has a function

as dietary fiber. Some dietary fibers are known to have an anti-obesity effect [57, 90]. Thus, the author conducted the studies to evaluate the anti-obesity effect of ESG (Chapters 2 and 3). It was found that ESG suppressed the increase of lipid accumulation in the adipose tissue and liver in diet-induced obese models. One distinct mechanism of the effect of ESG is to suppress fat absorption. It has been evident from the results of the increase in the amount of lipid excretion into feces and the decrease in the plasma TG level in a single dose study. Although, the detail of mechanism of absorption suppression effect is unknown, ESG did not affected lipase activity or micelle formation in vitro. Thus, the author consider the effect of ESG on the lipid absorption is not similar with that of indigestive dextrin which is well used in health beneficial food in Japanese market [96]. Effect on the intestinal microbiota could be considered as another mechanism of antiobesity effect of ESG. In this decade, it has become clear that intestinal bacteria are closely related to obesity [106]. Several probiotics and prebiotic materials are reported to exert anti-obesity effect by altering intestinal microbiota [107]. In Chapter 1, it was found that ESG reached the cecum maintaining the macromolecule, and increased Bifidobacterium and Lactobacillus in feces. There are several intestinal bacteria that are reported to be related to obesity, and the balance of the bacteria is considered to be important [106]. The author could not deeply consider the balance of intestinal microbiota, because the author conducted to analyze only six types of genus in this research. However, it is possible that increasing in Bifidobacterium and Lactobacillus are related to suppression of obesity by becoming dominant compared with other genus. In fact, it is reported that the visceral fat is decreased by administration of Bifidobacterium growing in the intestine [108]. The increase in the amount of SCFAs produced by intestinal bacteria and the change in ratio of SCFAs associated with the change in intestinal

microflora are also considered to be related to effectiveness, because certain reports have shown that SCFAs have anti-obesity effects [47, 55].

The progression of the aging society is becoming worldwide problem, and it is clear that the increase in patients with dementia will become a major social health issue in the near future. In recent years, it has begun to gradually understand the relationship between the intestinal function and the brain one. Specifically, it is becoming clear that intestinal microbiota and intestinal immunity are closely related to mental state and cognitive function. In this study, it became clear that ESG has a favorable effect on intestinal microbiota. On the one hand, in the previous studies, Kakutani et al. have reported that ESG has an enhancing effect of immunity in whole body through the activation of intestinal immune related cells [17, 18]. Therefore, the author hypothesized that ingestion of ESG could enhance the cognitive function of the brain. As a result of preliminary examination, ESG was expected to be effective. Thus the author conducted clinical trials in human (Chapter 4). Although, two subjects dropped out due to self-circumstances, no cases of health hazard due to ingestion of ESG was found. It was important first finding to confirm the safety of ESG in human clinical trial. Choice reaction time and learn memory accuracy were significantly improved by ingestion of ESG among the four items evaluated. On the other hand, there was no change in the simple reaction time and the accuracy of the one back task. These results suggested that ESG did not affect the whole brain function, such as by increasing the blood flow, but a part of the brain function, especially in high-order function. In addition, in an animal study that has been conducted additionally, it has been revealed that long term administration of ESG to mice significantly improves the test result of the Morris water maze (unpublished data). Although it was suggested that ESG has favorable effects on cognitive function, the detail

and mechanism of the function has been unknown. In the future, the detail and mechanism should be clarified by additional animal experiments and clinical tests in order to use ESG as a food material for improving cognitive function.

Various physiological effects of ESG have been clarified in this thesis and the previous studies. Glycogen is a substance widely present in the human body. Therefore, mechanisms for recognizing glycogen in the cells and tissues should have developed. The mechanisms could related to various physiological actions of ESG. In the future, the author expect further research will proceed and the role of glycogen *in vivo* becomes clear and then ESG will be the useful food material for promoting human health. The author will try to launch health beneficial food containing ESG in the near future.

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