Effect of oral supplementation with enzymatically synthesized glycogen (ESG) on cognitive function: a randomized, double-blind, placebo-controlled study

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ABSTRACT

Background: There is a well-established correlation between aging and decreasing cognitive performance in healthy adults. Furthermore, with increasing levels of stress in modern societies, cognitive decline is a growing concern. With our focus on these concens, we prepared enzymatically synthesized glycogen (ESG) from starch, and aimed to examine whether ESG supplementation improved cognitive functions in humans.

Methods: In a randomized, double-blind, placebo-controlled, cross-over trial, 40 healthy participants were administered 5.0 g of ESG or maltodextrin (placebo) beverages for 4 weeks, respectively. A washout period of 4 to 5 weeks was set between treatments. The primary endpoint

was the effect of orally administered ESG on cognitive function, which was assessed by using the CogHealth test battery. In addition, the fatigue VAS (visual analog scale) score and salivary levels of anti-fatigue factors (such as cortisol and secretory IgA) were determined.

Results: Two participants dropped out for personal reasons, therefore data for the remaining 38 subjects was analyzed. It was found that visual discrimination and long-term memory were significantly potentiated by the ingestion of ESG for 2-4 weeks compared with placebo treatment. On the other hand, the fatigue VAS score and salivary levels of anti-fatigue factors showed no significant differences between the ESG group and the placebo group.

Conclusions: Our study shows that oral administration of ESG significantly potentiates the cognitive performance of healthy volunteers. We speculate that glycogen is not only a vital energy source, but is also involved in enhancement of cognitive function.

Keywords: Glycogen, ESG, cognitive performance, CogHealth, long-term memory, visual discrimination.

INTRODUCTION

Glycogen is a multibranched polysaccharide in which glucose molecules are connected by α-1,4 glycoside bonds and the branches are connected by α-1,6 glycoside bonds. Its molecular weight ranges from 10^6 to 10^9 and it is a spherical nanoparticle with a diameter of 20-60 nm. Glycogen is found widely in nature, and is the major storage polysaccharide in animals, shellfish, and microorganisms [1]. In mammals, the largest glycogen reserves exist in the liver and skeletal muscles. The major functions of glycogen are to supply energy to the muscles and to release glucose into the blood from the liver [1]. Small quantities of glycogen are also present in other tissues, such as the brain, thymus, heart, skin, placenta, and leukocytes. It is unclear whether glycogen has other functions besides acting as an energy source [2-7].

In 1998, it was reported that glycogen extracted from scallops and oysters possessed immunomodulatory activity, while other glycogen preparations had little or no activity [8]. The authors suggested that these differing effects of glycogen might result from fine structural differences, which could depend on many factors such as the method of extraction or the source. To overcome the influence of such factors, we developed methods for enzymatic synthesis of homogeneous glycogen preparations [9]. These methods allow us to control several parameters of glycogen, such as the fine structure, molecular weight, and molecular size. Our enzymatically synthesized glycogen (ESG) has the same characteristics as glycogen from natural sources [10,11]. Using ESG, we have obtained the following findings: (i) glycogen activates macrophages, which

are important immune cells involved in antigen recognition, to produce nitric oxide and inflammatory cytokines such as interleukin-6 or interleukin-12 [12, 13], (ii) Toll-like receptor 2, which is a receptor involved in innate immunity, mediates the response of macrophages to glycogen [14], and (iii) orally administered ESG enhances systemic immune responses such as a tumor suppression effect, activation of natural killer cells in the spleen, and suppression of splenic sympathetic nervous activity [15]. These results suggest that glycogen not only has a role in maintaining blood glucose levels, but also has immunomodulatory effects. Moreover, it has been reported that orally administered ESG has prebiotic effects, including the improvement of lipid metabolism and modulation of enteric bacterial flora [16, 17]. Thus, various effects of orally administered ESG have been demonstrated.

It is well known that cognitive function gradually decreases with aging, while it declines rapidly in patients with dementia, such as Alzheimer's disease [18]. In addition, cognitive performance is decreased by alcohol intake, acute and chronic fatigue, and stress [19, 20]. Cognitive deficits in areas such as attention, memory, and reaction time significantly reduce one's quality of life. Therefore, improvement of cognitive performance is the 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 [21]. These subjects performed 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 stage of decline [21,22]. It was also reported that a flavonoid-rich extract of *Pinus radiata* bark improves cognitive function by enhancing immediate recognition and spatial working memory [23]. These reports suggest that oral administration of certain food components may enhance cognitive function. Accordingly, we examined whether the cognitive performance of healthy subjects was enhanced specifically by the oral administration of ESG. Using a double-blind, randomized, placebo-controlled, cross-over, study design we found that oral administration of ESG for 2-4 weeks significantly potentiated the cognitive performance of healthy volunteers. This included reaction times in the "choice reaction task" and accuracy in the "one card learning task". To the best of our knowledge, this is the first study investigating the effect of orally administered glycogen on cognitive brain function in humans. Our findings suggest that glycogen can potentially be used as a food component that improves cognitive function.

MATERIALS AND METHODS

Preparation of ESG

ESG (Bioglycogen^{TM}; Glico Nutrition Co., Ltd.) was prepared as described previously [9].   The α-1,6-glucosidic linkages of starch were completely digested with isoamylase which was derived

from *Pseudomonas amyloderamosa* to produce short chain amylose. After heating to inactivate the enzyme, recombinant amylomaltase, derived from *Thermus aquaticus* and the branching enzyme from *Aquifex aeolicus,* were added to the reaction mixture [24,25]. Since the *A. aeolicus* branching enzyme does not react efficiently with short chain amylose, amylomaltase was employed to elongate amylose and increase the efficiency of the branching reaction, allowing ESG to be formed from elongated amylose. In the present study, ESG was administered as a beverage. The composition of the ESG beverage and placebo beverage are shown in Table 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 15 mg of citric acid, 3.0 mg of sucralose, and 5.0g dextrin (ɑ-1,4/1,6 glucans) in place of the ESG. 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.

Table 1. Composition of the placebo and ESG beverages

	Placebo beverage	ESG beverage
ESG (mg)	0	5000
Maltodextrin (mg)	5000	0
Sucralose (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

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

Subjects

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

Table 2. Baseline characteristics of the subjects

Characteristic	Subjects					
	All (n=38)		Men (n=23)		Women (n=15)	
	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

*Two dropouts were excluded from this analysis.

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. 1).
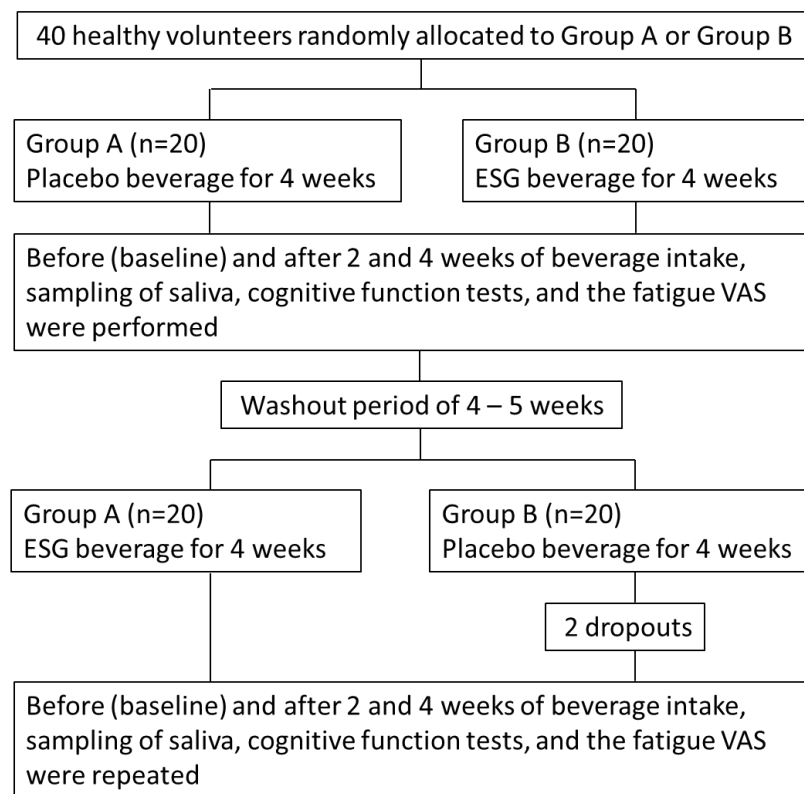
Fig.1 Flow diagram of the study

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 group B). All of the subjects, study administrators, and data processors were completely blind to the assignment of treatment groups.

During the first experimental period, the subjects ingested the assigned beverages once daily between 13:00 and 18:00 for 4 weeks; group A ingested the ESG beverage, while group B ingested the placebo beverage. After a washout period of 4 to 5 weeks, the beverages were reversed in the second experimental period. In each experimental period, cognitive testing and saliva sampling was completed three times; before treatment (baseline), after 2 weeks, and after 4 weeks of ingestion.

Prior to conducting the tests, subjects ate a meal of Japanese noodles between 12:00 – 12:30, after which any oral intake other than water was prohibited until 16:00. Subjects entered the test room at 16:00 and their level of fatigue was assessed using a simple horizontal visual analog scale (VAS), followed by the cognitive test for about 5 min. Subjects were allowed to rest for 20 minutes while sitting in a chair. A saliva sample was collected using a Salivette (Sartrious Co.) at 16:25, and cognitive testing began again at 16:30. In order to prevent influence of the ingested beverage on the testing day, each subject ingested ESG or placebo beverage after finishing the cognitive testing.

Cognitive testing

Cognitive performance was assessed using the CogHealth test. The battery of tests consist 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 first task is repeated again after the one card learning task (the last 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 seconds. Each time the card changes, the participant must indicate whether or not the new card is the same as the previous card by pressing either the "k" key or the "d" 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 seconds. 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 "k" key or the "d" key. When the response is correct, the card is moved to a single pack. This task is continued until the participant has made 42 correct responses or until the maximum time is reached.

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

Assessment of fatigue

The evaluation with VAS was carried out as soon as the subject entered the test room at 16:00. Subjects were instructed to make a mark on the fatigue VAS, which was a straight horizontal line 100 mm in length, according to their fatigue level. 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 minutes, 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 assay kits. Secretory IgA (sIgA) and cortisol concentrations were determined with the sIgA Salivary EIA Kit and Cortisol Salivary EIA Kit (Salimetrics Co.), while ɑ-amylase activity was measured with an assay from Oriental Yeast Co., Ltd..

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

We 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 3 as relative values compared with the baseline results. Reaction times in the "simple reaction task" and "working memory task" were significantly shorter after 4 weeks of ESG beverage intake compared with

baseline, while no such effect was observed with the placebo beverage. Also, the reaction time in the "choice reaction task" and accuracy in the "one card learning task" were significantly improved after 2 weeks and 4 weeks of ESG beverage intake, respectively, compared with the results for the placebo beverage. These findings suggest that ESG intake could improve several aspects of cognitive performance such as visual discrimination and memory.

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

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

Two dropouts were excluded from data analysis. *: $p<0.05$ *vs.* baseline. *N.S.*: not significant *vs.* placebo group. Data after 2 and 4 weeks of ESG intake 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.

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

Table 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 ingestion of the ESG and placebo beverages. Similarly, there was no significant difference of the VAS fatigue scores between the ESG and placebo beverages. Therefore, these results suggest that ingestion of ESG did not affect fatigue during daily life.

Table 4. Mean fatigue VAS score and salivary parameters at baseline and after ESG ingestion

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

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.

DISCUSSION

This randomized cross-over study was performed to investigate whether ESG enhanced cognitive performance, revealing that cognitive parameters such as visual discrimination and memory, assessed by CogHealth battery tests, were significantly improved by intake of ESG compared with

placebo (Table 3). Although, both ESG and the placebo used in this study (dextrin) are both (1→4)(1→6)-linked α-D-glucans, ESG contains far more α-1,6 branches than dextrin and is a high-density spherical molecule [11]. These features of ESG may result in minimal penetration of digestive enzymes such as α-amylase and α-glucosidase, resulting in enzymes gradually digesting ESG molecules from the periphery. Since enzymatic degradation of ESG is much slower than for dextrin, ESG may reach the distal small intestine [15, 17, 26]. 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 LD_{50} is > 2000 mg/kg/day [27], suggesting that it can potentially be used as a new food ingredient for improvement of cognitive performance.

It is common knowledge that cognitive performance is impaired by alcohol intake and by fatigue related to sleep disruption [19, 20]. Falleti and Maruff *et al*. both reported that the blood alcohol concentration of 0.05% or sustained wakefulness for 24 h could be similarly compared in the performance of cognitive function [19]. 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, we examined salivary levels of sIgA, cortisol, α-amylase, and the VAS fatigue score before the start of ESG intake and after ESG ingestion for 2 and 4 weeks. However, we found no significant difference of the salivary fatigue-related factors between subjects taking ESG and subjects taking placebo (Table 4). VAS scores also showed no significant difference between ESG and placebo. These findings indicated that the mechanism by which ESG improves cognitive performance is unrelated to an effect on fatigue.

A possible underlying mechanism on the effects of ESG may involve 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 impairment10 months after resection of the thymus [28]. In addition, the immune response of splenocytes is markedly reduced by aging in senescence accelerated mouse-prone 8 (SAMP8) mice. Furthermore, Lal *et al*. reported that immune senescence can induce deterioration of cognitive function [29]. They irradiated young mice to inactivate their immune systems, and then injected spleen and bone marrow cells obtained from aged mice with impaired learning, or cells from normal young mice. After injection of cells from aged mice, the young mice showed similar learning deficits to aged animals, while young mice receiving 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. Our previous studies have shown that oral ESG ingestion activates the immune system [15]. For example, we found that oral administration of ESG to mice activated $CD11b^+CD11c^+$ cells, such as macrophages and dendritic cells, in Peyer's patches, which are collections of lymphoid tissue 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 [15]. Thus, our 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 the present study. Investigations to clarify the mechanism by which ESG alters cognitive function are required in the future.

Competing interests: The authors are employees of Ezaki Glico Co., Ltd., however, this does not alter the author's adherence to the journal's policy.

Authors' contributions: All authors contributed as follows: Takashi Furuyashiki and Ryo Kakutani designed, carried out the experiments and co-wrote the manuscript. Mayumi Hokoyama-Fujita carried out the experiments. Kazuko Kato co-wrote the manuscript. Hiroshi Kamasaka and Takashi Kuriki gave advice in discussion and proofread the manuscript.

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