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Effects of Theracurmin® consumption on liver function, fatigue, and sleep: a randomized, double-blind, placebo-controlled, parallel-comparison study

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ABSTRACT

Objective: To evaluate the effects of the eight-week consumption of Theracurmin® on liver function, fatigue, and sleep.

Methods: This was a randomized, double-blind, placebo-controlled, parallel-comparison study involving 68 healthy Japanese adults. Subjects were allocated into either the active (Theracurmin®) or placebo group (n = 34 each) using a random number generator. Subjects consumed two capsules per day of either the active or placebo food for eight weeks. The primary outcome was the serum alanine aminotransferase (ALT) levels at eight weeks, whereas the secondary outcomes were the biomarkers of liver function, comparison of the percentages of improvement in liver function based on the decision criteria, OSA sleep inventory MA version (OSA-MA), and visual analog scale of fatigue.

Results: Each group included 33 subjects in the full analysis set. ALT levels in the per protocol set analysis, except for subjects drinking quantities of alcohol that increase the risk of lifestyle-related disease onset, showed a significant decrease in ALT compared to the placebo group (P < 0.05). The subjective symptom in the fatigue recovery factor of OSA-MA was significantly improved through the intervention (P < 0.05). The fatigue recovery

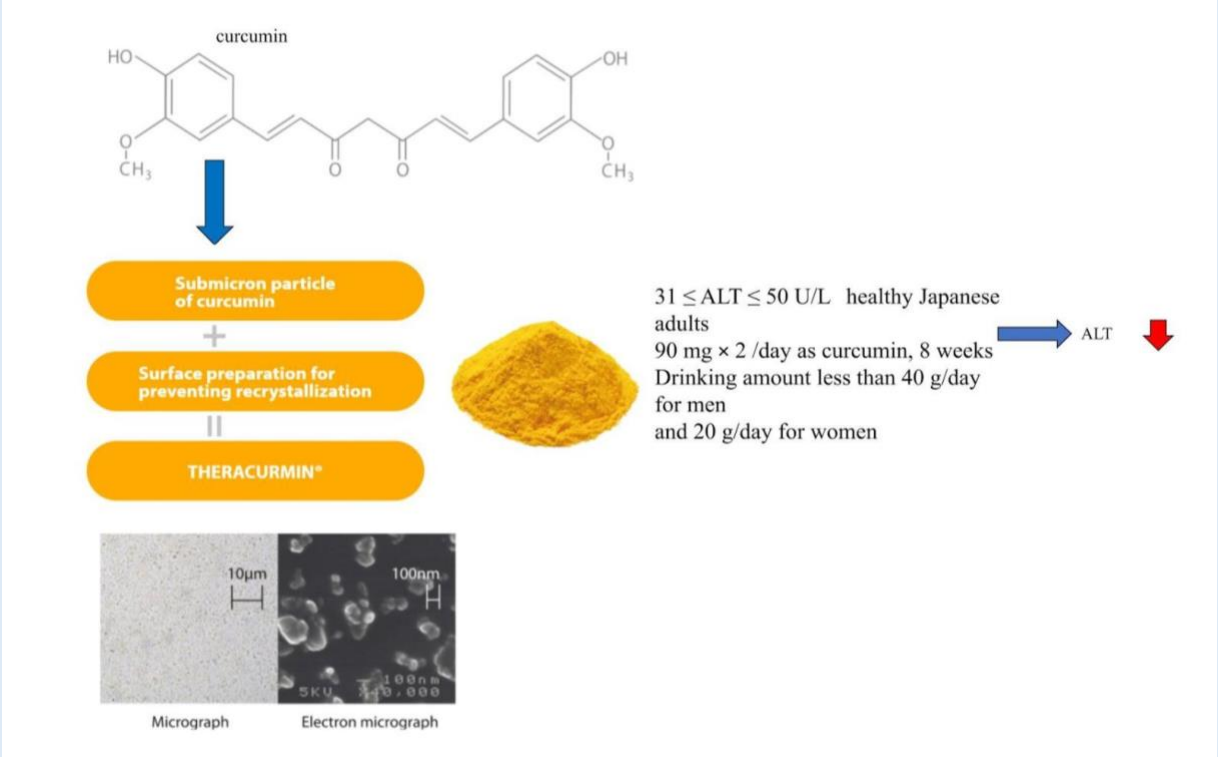
effect of Theracurmin® was prominent in the subjects aged ≥ 45 years, the age group defined by the Ministry of Health, Labour and Welfare as middle-aged and older persons in the Act on Stabilization of Employment of Elderly. No adverse event was observed.

Conclusions: These results suggest that the consumption of Theracurmin® or eight weeks improved liver function and fatigue recovery at awakening in healthy Japanese adults.

Trial registration: UMIN-CTR: UMIN000039319.

Foundation: Theravalues Corporation

Keywords: highly bioavailable curcumin, liver function, alanine aminotransferase (ALT), OSA sleep inventory MA version, fatigue recovery



INTRODUCTION

Medical advancements have led to a decrease in the number of new infections and cases of viral hepatitis, but fatty liver has been observed in approximately 30% of the examinees during health screening, and the prevalence is increasing [1]. Japanese are more likely to develop ectopic fat accumulation, such as visceral fat and fatty liver, when they are over nourished rather than subcutaneous fat [1]. This suggests that fatty liver is a lifestyle-related liver disease. Nonalcoholic fatty liver disease (NAFLD) is thought to progress to nonalcoholic steatohepatitis

(NASH) due to necroinflammatory changes induced by oxidative stress and insulin resistance [2,3]. The liver plays an important role in the maintenance of biological functions, such as the metabolism of nutrients and drugs, and in the detoxification of toxic substances. This means that preventing the deterioration of the liver function is important for maintaining and improving health.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase (γ-GT) are used to assess liver function. Degeneration or necrosis of hepatocytes causes the leakage of ALT and AST into the blood, and impaired excretion due to obstructive mechanisms in the liver and biliary system increases the production of γ-GT [4]. Particularly, ALT is found most abundantly in the liver compared to other organs and tissues in the body, and it is known to have high specificity for liver diseases [4]. Thus, there is a significant positive correlation between blood ALT levels and the amount of fat in the liver [5]. Unfavorable lifestyle habits such as excessive alcohol consumption, smoking, overeating, and low physical activity have been reported to increase the risk of high levels of ALT, γ-GT, and the inflammatory marker high-sensitivity C-reactive protein (CRP) [6]. Furthermore, decreasing ALT may be associated with the prevention of metabolic syndrome and longevity [7]. Therefore, maintaining a low level of ALT is important in order to prevent damage to hepatocytes and maintain liver function.

Several food components have been identified as being useful in preserving the liver function [8], one of which is curcumin, the major curcuminoids found in turmeric rhizome. Curcumin has been attracting attention for its hepatoprotective function due to its many pharmacological activities. The chemical structure of curcumin contains a conjugated double bond, which functions as an electron donor to counteract the formation of reactive oxygen species in many redox reactions [9], thus exhibiting antioxidant effects. In addition, curcumin has been shown to have antioxidant, anti-inflammatory, antitumor, antibacterial, and antiviral effects [10–13]. In fact, clinical trials have reported that curcumin can be used to treat cardiovascular, inflammatory, metabolic, neurological, skin, and liver diseases [14]. However, it is known that curcumin has a low bioavailability, and it is necessary to combine it with natural or synthetic compounds to increase its bioavailability and obtain the desired efficiency [14].

Theracurmin®, the active food in this study, is a highly absorbable oral formulation of curcumin, which has been shown to have a significantly higher bioavailability than conventional curcumin in animals and humans [15–17]. A previous clinical study using Theracurmin®, the same food in this study, reported that the intake of 30 mg, 60 mg, and 90 mg of Theracurmin® twice daily for one month led to a dose-dependent decrease in AST, ALT, and γ-GT [18]. However, there have been no placebo-controlled clinical trials in humans to date. Therefore, this study aimed to examine the effect of Theracurmin® on liver function using a placebo as a control. In addition, it has been reported that liver diseases cause fatigue, depression, and sleep disturbances [19]. In another previous clinical study, taking Theracurmin® was reported to improve fatigue in daily life, lethargy

upon waking, and awakening in women [20]. Thus, we also evaluated the effect of Theracurmin® on sleep and fatigue.

METHOD

Study design: This was a randomized, double-blind, placebo-controlled, parallel-comparison study, and allocation based on a 1:1 ratio. The study protocol was approved by the Ethics Committee of Takara Clinic (Medical Corporation Seishinkai, Tokyo, Japan; Approval ID: 2001-1912-SR01-01; January 24, 2020) and registered at the University Hospital Medical Information Network Clinical Trial Registry (Registry no. UMIN000039319). This study was conducted in accordance with the principles of the Declaration of Helsinki (2013) and ethical guidelines for medical and health research involving human subjects in Japan with complete consideration of medical ethics.

Subjects: The inclusion criterion was defined as healthy Japanese adult subjects. Exclusion criteria were defined as follows: (a) a medical history or current treatment for a malignancy, heart failure, or myocardial infarction; (b) a history of severe liver disorder (viral hepatitis, drug-induced liver injury, cirrhosis) or currently suspected of having a liver disorder (a positive test result on hepatitis screening, etc.); (c) use of a pacemaker or an implantable cardioverter defibrillator; (d) current treatment for cardiac arrhythmia, hepatic, renal, or cerebrovascular disease; rheumatism; diabetes mellitus; hyperlipidemia; hypertension; or other chronic diseases; (e) a history of diseases related to the biliary tract or are currently suspected of having diseases related to the biliary tract; (f) daily consumption of "foods for specified health uses," "foods with function claims," or other functional foods/beverages; (g) currently taking medicines and/or herbal medicines, particularly using anticoagulants such as Warfarin; (i) being pregnant, lactating, or planning to become pregnant; (j) enrollment in other clinical trials within the last three months before the agreement to participate in this trial or planning to participate in another trial during this trial; and (k) ineligible to participate in the study based on the evaluation of the principal physician. The subjects were recruited via the website (https://www.go106.jp/) operated by ORTHOMEDICO Inc. (Tokyo, Japan). The study protocols were comprehensively explained to all subjects at the office of ORTHOMEDICO Inc. Furthermore, all subjects signed an informed consent prior to their participation in the study. No subject was part of the sponsors or funding companies.

Intervention: Subjects were asked to consume either one active (Theracurmin®, 90 mg per capsule as curcumin; the active group) or placebo capsule (excipients only; the placebo group) with water 10 to 15 minutes before breakfast and dinner for eight weeks. The Ethics Committee declared both capsules have identical color, odor, and flavor.

Outcomes: The schedule of this study is shown in Table 1. The efficacy except for the visual analog scale (VAS) of fatigue and safety assessment were conducted at screening (examination before consumption: Scr), then four and eight weeks after

intake (4wks and 8wks, respectively). The examinations were conducted at Takara Clinic (Medical Corporation Seishinkai, Tokyo, Japan). The reference values for blood test items were based on the information disclosed by LSI Medience Corporation (Tokyo, Japan). Only ALT values were taken from the information published by the Japan Society of Ningen Dock [21].

(1) Primary outcome: measured value of serum ALT levels at 8wks

Approximately 13 mL of venous blood was collected from each subject for hematological tests. Hematological tests were conducted to assess the ALT level. The ALT level was measured by LSI Medience Corporation.

(2) Secondary outcomes

1) The ratio of the number of subjects who achieved the decision criterion 1 after the intervention

The decision criterion 1 was defined as follows: the value of ALT at 8wks was 30 U/L or less (the reference range) [21]. The number of subjects who achieved the decision criterion 1 after the intervention was compared between both groups.

2) The ratio of the number of subjects who achieved the decision criterion 2 after the intervention

The decision criterion 2 was defined as follows: (1) ALT levels between 31 U/L and 40 U/L (the caution range [21]) at Scr decreased to 30 U/L or less at 8wks, or (2) ALT levels between 41 U/L and 50 U/L (the caution range [21]) at Scr decreased to 40 U/L or less at 8wks. The number of subjects who achieved the decision criterion 2 after the intervention was compared between groups.

3) ALT, AST, γ-GT

In hematological tests, subjects were assessed for the following: "The measured values of serum ALT at 4wks," "The change of serum ALT between Scr and at 4wks and 8wks," "The measured values of AST and γ-GT at 4wks and 8wks," and "The change of serum AST and γ-GT between Scr and at 4wks and 8wks." It was measured in a manner similar to the primary outcome.

4) OSA sleep inventory MA version (OSA-MA) [22,23]

Sleep state was evaluated using OSA-MA. Subjects were asked to fill out a questionnaire two days before the examinations and on the day of the examinations. In the questionnaire, the measured values and change in score of five factors about "sleepiness on rising (Factor I)," "initiation and maintenance of sleep (Factor II)," "frequent dreaming (Factor III)," "feeling of refreshment (Factor IV)" and "sleep length (Factor V)," and question score of 16 items was evaluated. For each evaluation item, the Zc value was calculated for each day, and the average value for three days was used as the measured value at each time point. The higher

questionnaire scores indicated a better sleep condition.

5) VAS of fatigue [24,25]

Using the VAS of fatigue recommended by the Japanese Society of Fatigue Science, we evaluated the measured values and changes in the VAS of fatigue at 2wks, 4wks, 6wks, and 8wks. It was performed according to the method of the Japanese Society of Fatigue Science [24,25].

(3) Safety evaluation

Safety evaluations were assessed by physical examination, urinalysis, and blood analysis. The subjects' weight, body mass index, body fat percentage, systolic and diastolic blood pressures, and pulse rate were measured as the physical examination items. Height was only measured at Scr to calculate the body mass index.

In the urinalysis, urine samples were collected to evaluate the levels of protein, glucose, urobilinogen, bilirubin, ketone bodies, pH, and occult blood. The collected samples were entrusted to LSI Medience Corporation (Tokyo, Japan), and each item was evaluated in accordance with the global standard.

Hematological tests were performed to assess the following: leukocyte count, erythrocyte count, hemoglobin, hematocrit, platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and white blood cell differential count (i.e., numbers of neutrophils, lymphocytes, monocytes, eosinophils, and basophils). For the biochemical tests, we evaluated the following: alkaline phosphatase, lactate dehydrogenase, leucine aminopeptidase, total bilirubin, direct bilirubin, indirect bilirubin, cholinesterase, total protein, urea nitrogen, creatinine, uric acid, creatine kinase, calcium, serum amylase, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, glycoalbumin, serum iron, sodium, potassium, chloride, inorganic phosphorus, glucose, and hemoglobin A1c. In addition, nonspecific immunoglobulin E was only measured at Scr. The level of each parameter was measured by LSI Medience Corporation.

All subjects were asked to complete a medical questionnaire to allow for the understanding of their health conditions at each examination. In addition, subjects were asked to keep a daily record of the consumption of the test food, health conditions, use of medications, and lifestyle.

Table 1. Schedule of enrollment, intervention, and assessments.

					Intervention Period				
	Examination	Screening (Scr)	Enrollment	Allocation	Start intake (0w)	Two weeks after the start of the test-food consumption (2wks)	Four weeks after the start of the test-food consumption (4wks)	Six weeks after the start of the test-food consumption (6wks)	Eight weeks after the start of the test-food consumption (8wks)
ENROLLMENT:									
Eligibility screen	●		●						
Informed consent	●								
Other procedures	●								
Allocation				●					
INTERVENTIONS:									
Active group					←	—	—	—	→
Placebo group					←	—	—	—	→
ASSESSMENTS:									
VAS of fatigue		●				●	●	●	●
OSA sleep inventory MA version		●					●		●
Physical examination	●	●					●		●
Urinalysis		●					●		●
Blood test		●					●		●
Dietary survey		●					●		●
Medical questionnaire		●					●		●
Daily record					←	—	—	—	→

Closed circles (●) display the execution timing of each item

Sample size: The sample size was calculated with an assumption that the difference in the measured values of ALT between the active and placebo groups at 8wks was large. As suggested by Cohen (1992), the sample size was calculated with an assumed effect size (d) of 0.80[26], significance level (α) of 0.05, and statistical power (1−β) of 0.90, leading to 34 subjects per group (68 subjects in total).

Selection, randomization, and blinding: Of 204 subjects who signed the informed consent forms, 68 eligible subjects who were considered appropriate for the study were selected by the physician. Inclusion criteria were defined as follows: (1) eligible to participate in the study based on the evaluation of the principal physician; (2) serum ALT ≥ 31 U/L and ≤ 50 U/L.

Test foods were provided by Theravalues Corporation (Tokyo, Japan) to the contract research organization, ORTHOMEDICO Inc. The allocation was performed according to a computer-generated randomization list by an allocation controller, who was not directly involved in this study. Based on allocation adjustment factors such as sex, age, and measured values of serum ALT at Scr, the subjects were equally, but randomly assigned to either the active or placebo group (n = 34 per group). After declaring that the test foods could not be distinguished, the allocation table with the coded test foods was provided only to the person in charge of the shipping, who sent the test foods to each subject according to the table. The sponsors, principal investigator, sub-investigators, entire contract research organization staff (i.e., the director of the trial, the director of trial conduction, the person in charge of monitoring, the director and staff of statistical analysis, and the person in charge of shipping), medical institution staff, institutional review board members, contract laboratory, and others who were related to this study were not aware of the group assignments. The allocation controller locked the allocation table until the key opening day.

Statistical analysis: All statistical analyses in this study were two-sided, and the significance level was set at 5% with no adjustment for multiple comparisons. Data analyses were performed using Windows SPSS version 23.0 (IBM Japan, Ltd., Tokyo, Japan) and Microsoft Excel 2013 (Microsoft Japan Co., Ltd., Tokyo, Japan).

Subjects' background data were demographically aggregated by the enrolled and analyzed subjects. Sex was compared using χ^2 test, and age, height, and nonspecific IgE (radioimmunosorbent test) were compared using Student's t-test.

The primary outcomes and secondary outcomes (except for the comparison of the number of subjects), and the safety assessment items (except for urinalysis) were presented as means and standard deviations. The data of each item at Scr was set as the baseline. The changes in the value were identified by subtracting the baseline values from the measured values at 4wks and 8wks. The baseline and the changes in the value were analyzed using Student's t-test. The measured values of the primary outcome and the secondary outcomes (except for the comparison of the number of subjects) at 4wks and 8wks were analyzed using post-hoc comparisons with linear mixed model, with the baseline values utilized as covariates and time, group, group–time interaction, and subject as factors. In addition, we analyzed the data of the safety assessment items at 4wks and 8wks using the analysis of covariance (ANCOVA) with the baseline values as a covariate and the group as factors. Furthermore, changes in the value (4wks−Scr, 8wks−Scr) were analyzed using post-hoc comparisons with a linear mixed model, with

time, group, group–time interaction, and subject as factors. To compare percentages of the improvement in liver function, logistic regression analysis with factors of group, sex, age, and ALT at baseline was performed to evaluate the odds ratio (OR), 95% confidence interval, and *P* value to the placebo group. Furthermore, urinalysis data were assigned codes wherein "1" was identified as within the normal range and "0" was identified as outside the normal range.

RESULTS

Analysis set: The study flowchart and subject disposition are illustrated in Fig. 1. We recruited subjects for this study from January 30, 2020, to June 6, 2020, and conducted it from February 5, 2020, to August 22, 2020.

For this study, we planned to use the ITT, the per-protocol set (PPS), and the full analysis set (FAS) to confirm our analysis. Since the primary outcome of this study is ALT, a marker that is highly sensitive to changes in daily life, we thought that in some cases it would be necessary to check for subjects who had significant lifestyle changes before the key opening. At the case review meeting before the key opening, we checked for subjects who dropped out or violated compliance during the study period. One subject in the active group and one in the placebo group did not come to the hospital after 4-weeks of the examination. Additionally, in this study, some subjects consumed less alcohol before allocation but consumed excessive alcohol throughout the intervention period. The results of previous epidemiological studies have shown that the risk of several health problems associated with alcohol consumption, such as cancer, hypertension, cerebral hemorrhage, and dyslipidemia increases almost linearly with the average daily alcohol consumption [28–30]. According to the World Health Organization guidelines, the threshold for increased risk of alcohol-related problems is drinking more than 40 g/day for men and 20 g/day for women [31]. Moreover, in Japan, the Ministry of Health, Labor, and Welfare (MHLW) has published the "Health Japan 21 (the second term)" as a basic policy to promote the overall health of the population, defining the amount of alcohol consumption (pure alcohol intake) that increases the risk of lifestyle-related diseases as an average of 40 g or more per day for men and 20 g or more for women, suggesting that the population should drink with moderation [27]. However, if the subjects who drank alcohol in amounts that increased the risk of lifestyle-related diseases were included in the analysis, it could not be assumed that the conditions for confirming the effects of the test food were in place for assessing liver function. Therefore, in accordance with "Health Japan 21 (the second term) [27]," the principal physician excluded the four subjects whose pure alcohol intake averaged 40 g or more per day for men and 20 g or more for women throughout the intervention from the analysis and conducted the PPS analysis.

The final efficacy analysis subjects for the liver function markers, including the primary outcome, were the PPS, and 31 subjects (25 men and 6 women) in the active group and 31 subjects (26 men and 5 women) in the placebo group were included. As for the other outcomes, the analysis population was the FAS, representing the analysis population that excludes only subjects that have never come to the clinic after allocation, and 33 (27 men and 6 women) in the active group and 33 (27 men and 6 women) in the placebo group. The subjects of the safety analysis were the safety analysis population, which was the same as the FAS. The ITT included all subjects in this study. The background and age distribution of the study subjects are shown in Table 2. There was no significant difference between the background factors of both groups.

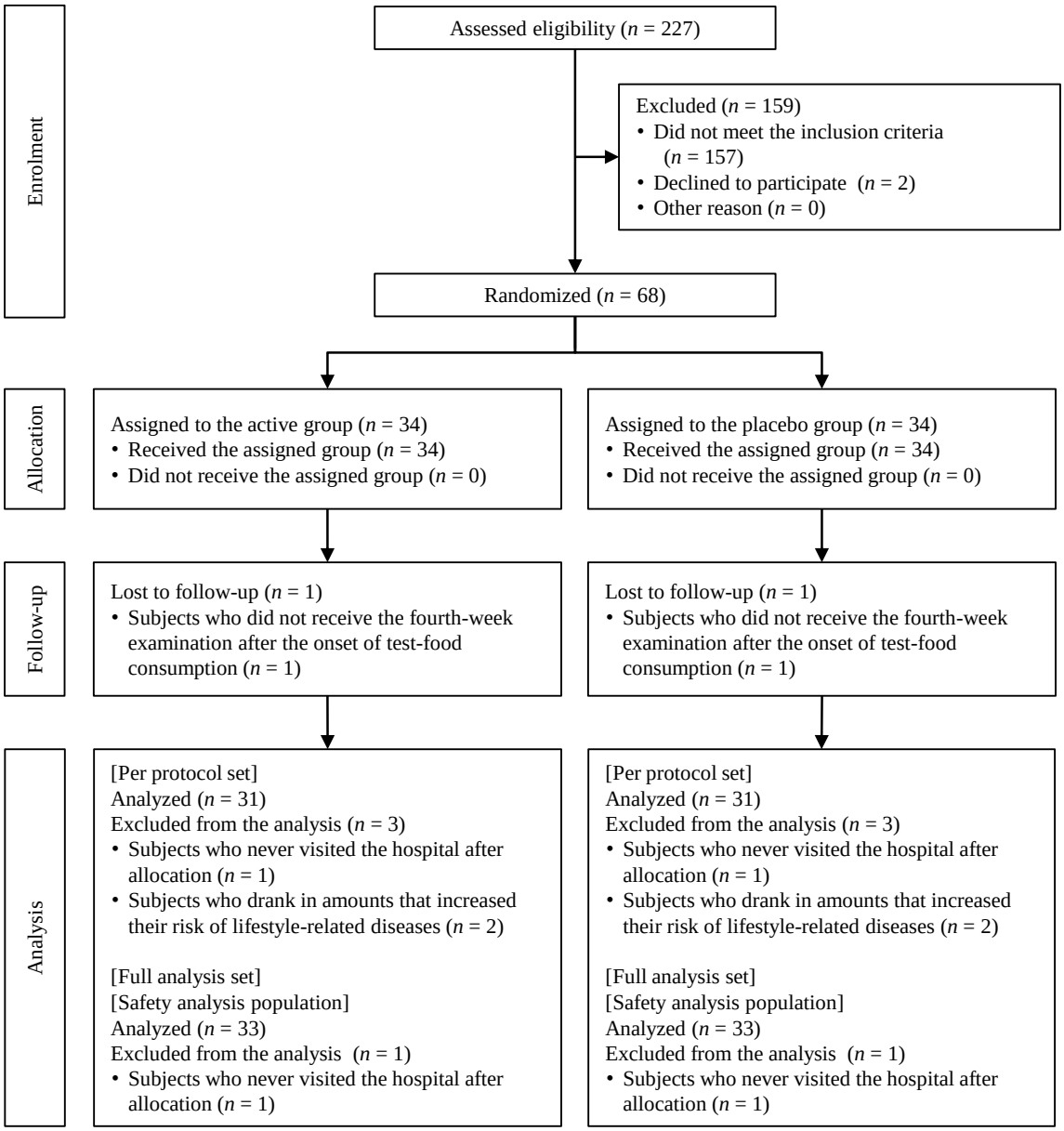


Figure 1. Flowchart of participants in this study.

Table 2. Subjects' background information.

	Active group		Placebo group		*P* value
	Mean (Men)	SD (Women)	Mean (Men)	SD (Women)	
Allocated subjects (Active group *n* = 34, Placebo group *n* = 34)					
Sex	27 (79.4%)	7 (20.6%)	27 (79.4%)	7 (20.6%)	> 0.999
Age (years)	44.9	11	44.4	10.3	0.86
Body weight (kg)	73.2	13.6	70.2	9.7	0.29
Non-specific IgE (IU/mL)	297.1	346.3	156.9	325.5	0.09
Full analysis set; FAS and Safety analysis population; SAF (Active group *n* = 33, Placebo group *n* = 33)					
Sex	27 (81.8%)	6 (18.2%)	27 (81.8%)	6 (18.2%)	> 0.999
Age (years)	44.9	11.2	44.3	10.4	0.81
Body weight (kg)	73.8	13.4	70.7	9.4	0.28
Non-specific IgE (IU/mL)	306.1	347.6	160.2	330	0.09
Per Protocol Set; PPS (Active group *n* = 31, Placebo group *n* = 31)					
Sex	25 (80.6%)	6 (19.4%)	26 (83.9%)	5 (16.1%)	> 0.999
Age (years)	45.6	10.8	44.5	10.1	0.67
Body weight (kg)	73.5	13.7	70.4	9.5	0.30
Non-specific IgE (IU/mL)	322.6	352.3	167.5	339.5	0.08

The data of sex is indicated as the number of subjects and as a percentage of each group and assessed by the chi-square test for the between-group comparisons. The other data are presented as the number of subjects, or the mean and standard deviation (SD) and evaluated using Student's *t*-test for the between-group comparisons.

ALT, AST, γ-GT, and AST/ALT ratio: In the ITT dataset, there were no statistical differences between groups (data not shown).

The results of ALT, AST, γ-GT, AST/ALT ratio in PPS are shown in Tables 3-1 to 3-3. The trend of ALT in PPS is also shown in Fig. 2. The data at 4wks and 4wks−Scr is shown in Appendices 1-1 to 1-3.

ALT at 8wks was 29.7 ± 10.8 U/L in the active group and 37.8 ± 17.8 U/L in the placebo group and was significantly lower in the active group than in the placebo group (P = 0.036). ALT at 8wks−Scr was −8.3 ± 11.2 U/L in the active group and −1.1 ± 15.1 U/L in the placebo group, which was significantly lower in the active group than in the placebo group (P = 0.034). Regarding the ratio of the number of subjects who achieved the decision criteria, there was no significant difference between the groups (data not shown).

Table 3-1. ALT, AST, γ-GT, AST/ALT ratio (PPS)

	Reference range	Unit		Scr			8wks			8wks−Scr		
				Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value
ALT	≤30	U/L	Active group (*n* = 31)	38.1	5.5	0.57	29.7	10.8	0.036*	−8.3	11.2	0.034*
			Placebo group (*n* = 31)	38.9	6.1		37.8	17.8		−1.1	15.1	
AST	10-40	U/L	Active group (*n* = 31)	30.2	7.6	0.79	28.0	8.5	0.31	−2.2	7.7	0.29
			Placebo group (*n* = 31)	29.7	5.8		30.1	10.0		0.4	8.5	
γ-GT	Male: ≤80 Female: ≤30	U/L	Active group (*n* = 31)	79.8	57.1	0.30	78.8	68.0	0.28	−0.9	32.3	0.38
			Placebo group (*n* = 31)	65.7	49.4		71.6	58.0		5.9	28.6	
AST/ALT ratio	-	-	Active group (*n* = 31)	0.8	0.2	0.66	1.0	0.3	0.07	0.2	0.3	0.07
			Placebo group (*n* = 31)	0.8	0.2		0.9	0.3		0.1	0.2	

The data are presented as the mean and standard deviation (SD). The baseline (Scr) values are evaluated using Student's *t*-test, the measured values are evaluated using Post-hoc comparisons with linear mixed model, with the baseline values utilized as covariates and time, group, group–time interaction, and subject as factors, and the changes are evaluated using Post-hoc comparisons with linear mixed model, with time, group, group–time interaction, and subject as factors.

*: $P < 0.05$ vs. the placebo group.

Table 3-2. ALT, AST, γ-GT, AST/ALT ratio (PPS / Men)

	Reference range	Unit		Scr			8wks			8wks−Scr		
				Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value
ALT	≤30	U/L	Active group (*n* = 25)	38.4	5.4	0.79	30.5	11.5	0.05	−8.0	11.3	0.05
			Placebo group (*n* = 26)	38.0	5.4		37.2	18.4		−0.8	15.7	
AST	10-40	U/L	Active group (*n* = 25)	29.4	6.3	0.82	27.6	8.5	0.19	−1.8	5.4	0.19
			Placebo group (*n* = 26)	29.1	5.0		30.2	10.5		1.1	8.4	
γ-GT	≤80	U/L	Active group (*n* = 25)	77.0	53.3	0.55	76.6	62.1	0.41	−0.4	34.3	0.45
			Placebo group (*n* = 26)	68.3	50.0		74.7	59.7		6.3	31.0	
AST/ALT ratio	-	-	Active group (*n* = 25)	0.8	0.1	0.83	1.0	0.3	0.25	0.2	0.3	0.24
			Placebo group (*n* = 26)	0.8	0.2		0.9	0.3		0.1	0.2	

The data are presented as the mean and standard deviation (SD). The baseline (Scr) values are evaluated using Student's *t*-test, the measured values are evaluated using Post-hoc comparisons with linear mixed model, with the baseline values utilized as covariates and time, group, group–time interaction, and subject as factors, and the changes are evaluated using Post-hoc comparisons with linear mixed model, with time, group, group–time interaction, and subject as factors.

Table 3-3. ALT, AST, γ-GT, AST/ALT ratio (PPS / Women)

	Reference range	Unit		Scr				8wks				8wks−Scr		
				Mean	SD	*P* value		Mean	SD	*P* value		Mean	SD	*P* value
ALT	≤30	U/L	Active group (*n* = 6)	36.5	6.5	0.15		26.7	7.1	0.31		−9.8	11.6	0.44
			Placebo group (*n* = 5)	43.4	8.2			41.0	15.6			−2.4	12.8	
AST	10-40	U/L	Active group (*n* = 6)	33.3	11.9	0.98		29.7	9.3	1.00		−3.7	14.5	0.99
			Placebo group (*n* = 5)	33.2	9.0			29.6	7.8			−3.6	8.3	
γ-GT	≤30	U/L	Active group (*n* = 6)	91.5	75.6	0.34		88.3	95.4	0.39		−3.2	24.6	0.67
			Placebo group (*n* = 5)	51.8	48.7			55.6	51.3			3.8	10.7	
AST/ALT ratio	-	-	Active group (*n* = 6)	0.9	0.1	0.12		1.1	0.3	0.13		0.2	0.2	0.08
			Placebo group (*n* = 5)	0.8	0.1			0.8	0.1			0.0	0.2	

The data are presented as the mean and standard deviation (SD). The baseline (Scr) values are evaluated using Student's *t*-test, the measured values are evaluated using Post-hoc comparisons with linear mixed model, with the baseline values utilized as covariates and time, group, group–time interaction, and subject as factors, and the changes are evaluated using Post-hoc comparisons with linear mixed model, with time, group, group–time interaction, and subject as factors.

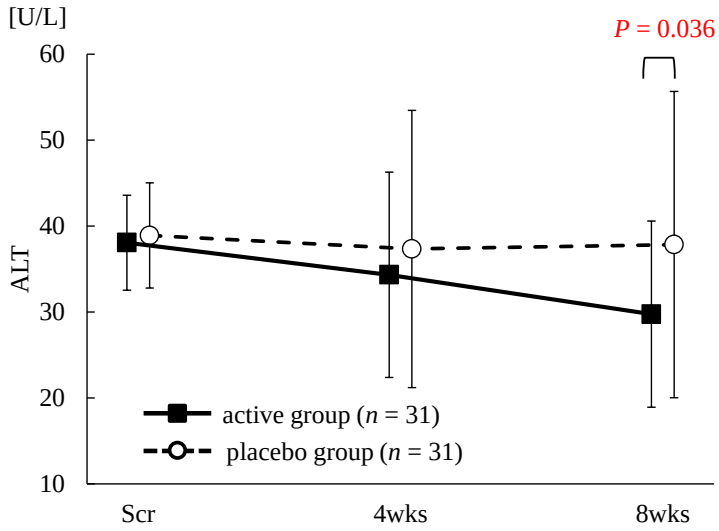


Figure 2. Changes in ALT (PPS) level.
The data are presented as the mean and standard deviation (SD).

OSA sleep inventory MA version (OSA-MA): In ITT dataset, question 11 (feelings) in 8wks−Scr was significantly higher in the active group than in the placebo group. {active group, 0.7 ± 6.4 point; placebo group, −2.9 ± 7.6 (*P* = 0.048)}.

Table 4-1 shows the results of the FAS analysis, and Table 4-2 shows the results of the OSA-MA for the subgroup analysis of those aged 45 years or older, who are defined as middle-aged or older in the FAS by the Ordinance of the MHLW of the Act on the Stabilization of Employment of Elderly Persons. In addition, the data at 4wks and 4wks−Scr is shown in Appendices 2-1 to 2-3, and the results of each question's score of OSA-MA for the subgroup analysis of subjects under 45 years in the FAS are shown in Appendix 2-4.

In the FAS analysis, question 11 (feelings) in 8wks−Scr was significantly higher in the active group than in the placebo group. {active group, 0.8 ± 6.5 point; placebo group, −2.9 ± 7.7 (*P* = 0.048)}.

In the subgroup analysis of subjects aged 45 years and older in FAS, question 12 (dreaming) at Scr determined that the active group was significantly higher than the placebo group {active group, 26.2 ± 5.0 point; placebo group, 21.6 ± 7.7 (*P* = 0.040)}. In addition, question 14 (answering ability) in Scr determined that the active group was significantly lower than the placebo group {active group, 19.7 ± 6.5 point; placebo group, 24.6 ± 5.4 (*P* = 0.018)}. The feeling refreshed score in 8wks−Scr was that the active group was significantly higher than the placebo group {active group, 0.9 ± 5.3 point; placebo group, −3.0 ± 7.2 (*P* = 0.047)}. Also, question 5 (dullness) in 8wks−Scr was that the active group was significantly higher than the placebo group {active group, 1.6 ± 7.0 point; placebo group, −3.3 ± 7.1; EMM 4.9 point (*P* = 0.021)}.

In the subgroup analysis of subjects aged under 45 years in FAS, question 7 (dozing off) at Scr was that the active group was significantly higher than the placebo group {active group, 24.6 ± 5.5 point; placebo group, 18.2 ± 8.7 (*P* = 0.023)}. The sleep length scores of OSA-MA at 8wks {active group, 24.9 ± 5.9 point; placebo group, 20.1 ± 7.8 (*P* = 0.034)} and 8wks−Scr {active group, 0.7 ± 5.7 point; placebo group, −3.7 ± 6.3 (*P* = 0.044)} were significantly higher in the active group than in the placebo group. In addition, 8wks−Scr {active group, −2.3 ± 7.1 point; placebo group, 3.8 ± 5.4 (*P* = 0.026)} were significantly lower in the active group than in the placebo group.

Table 4-1. OSA-MA (FAS)

	Unit		Scr			8wks			8wks−Scr		
			Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value
Sleepiness on rising	point	Active group (*n* = 33)	21.0	5.7	0.55	20.6	5.1	0.45	−0.3	6.1	0.34
		Placebo group (*n* = 33)	21.8	5.6		20.0	6.2		−1.8	6.7	
Initiation and maintenance of sleep	point	Active group (*n* = 33)	20.0	4.9	0.64	21.0	4.8	0.23	1.0	4.6	0.22
		Placebo group (*n* = 33)	20.6	6.0		20.1	4.7		−0.5	5.3	
Frequent dreaming	point	Active group (*n* = 33)	24.5	5.9	0.25	23.1	6.0	0.68	−1.4	6.2	0.33
		Placebo group (*n* = 33)	22.9	5.8		22.8	5.7		0.0	6.2	
Feeling refreshed	point	Active group (*n* = 33)	21.4	5.6	0.59	21.0	5.1	0.21	−0.4	6.4	0.19
		Placebo group (*n* = 33)	22.2	5.3		19.5	5.5		−2.6	6.6	
Sleep length	point	Active group (*n* = 33)	23.2	4.2	0.58	23.7	5.4	0.07	0.5	4.6	0.06
		Placebo group (*n* = 33)	23.9	5.9		21.7	6.6		−2.2	6.3	
Question 1 (remaining fatigue)	point	Active group (*n* = 33)	21.2	6.3	0.89	19.8	5.9	0.39	−1.4	7.8	0.59
		Placebo group (*n* = 33)	21.0	6.3		18.5	6.1		−2.4	8.0	
Question 2 (concentration ability)	point	Active group (*n* = 33)	21.8	7.2	0.73	21.7	5.9	0.33	−0.1	7.8	0.34
		Placebo group (*n* = 33)	22.4	7.6		20.5	6.7		−1.9	7.7	
Question 3 (deepness of sleep)	point	Active group (*n* = 33)	17.7	6.3	0.15	19.0	7.3	0.61	1.3	7.8	0.20
		Placebo group (*n* = 33)	20.1	7.0		19.1	5.6		−1.0	7.4	
Question 4 (relaxing)	point	Active group (*n* = 33)	20.4	6.1	0.61	21.2	5.5	0.36	0.8	8.1	0.79
		Placebo group (*n* = 33)	19.6	7.3		19.8	5.7		0.2	8.8	
Question 5 (dullness)	point	Active group (*n* = 33)	20.2	5.6	0.67	19.5	6.4	0.34	−0.6	8.3	0.31
		Placebo group (*n* = 33)	20.8	5.8		18.3	5.9		−2.5	6.9	
Question 6 (appetite)	point	Active group (*n* = 33)	26.5	7.3	0.72	24.7	8.4	0.19	−1.7	5.9	0.17
		Placebo group (*n* = 33)	27.1	7.8		23.0	9.0		−4.1	7.5	

	Unit		Scr			8wks			8wks−Scr		
			Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value
Question 7 (dozing off)	point	Active group (*n* = 33)	22.5	6.2	0.38	22.4	5.8	0.91	−0.1	6.5	0.65
		Placebo group (*n* = 33)	20.9	8.2		21.7	7.6		0.8	7.4	
Question 8 (clearness of thought)	point	Active group (*n* = 33)	20.2	6.7	0.36	20.3	6.6	0.21	0.1	6.7	0.12
		Placebo group (*n* = 33)	21.7	7.0		18.9	7.7		−2.8	8.4	
Question 9 (nightmares)	point	Active group (*n* = 33)	26.0	5.9	0.41	25.2	5.3	0.83	−0.8	5.3	0.49
		Placebo group (*n* = 33)	24.8	6.0		24.9	5.4		0.1	6.0	
Question 10 (quality of sleep)	point	Active group (*n* = 33)	17.7	7.5	0.56	19.8	6.1	0.16	2.1	7.6	0.14
		Placebo group (*n* = 33)	18.8	7.9		18.3	7.2		−0.5	6.3	
Question 11 (feelings)	point	Active group (*n* = 33)	23.0	6.4	0.24	23.7	5.9	0.11	0.8	6.5	0.048*
		Placebo group (*n* = 33)	24.8	5.8		21.8	6.3		−2.9	7.7	
Question 12 (dreaming)	point	Active group (*n* = 33)	23.1	8.0	0.27	21.0	8.3	0.66	−2.1	8.9	0.35
		Placebo group (*n* = 33)	21.0	7.4		20.8	8.2		−0.2	8.0	
Question 13 (waking at night)	point	Active group (*n* = 33)	21.4	7.3	0.45	22.0	6.9	0.37	0.6	6.3	0.24
		Placebo group (*n* = 33)	22.6	5.7		21.2	5.6		−1.4	7.3	
Question 14 (answering ability)	point	Active group (*n* = 33)	21.5	6.9	0.20	19.3	6.3	0.82	−2.2	6.3	0.74
		Placebo group (*n* = 33)	23.5	5.5		20.8	7.7		−2.7	6.4	
Question 15 (sleeping time)	point	Active group (*n* = 33)	19.9	5.4	0.64	22.7	6.0	0.07	2.8	6.2	0.07
		Placebo group (*n* = 33)	20.6	7.1		20.4	7.2		−0.2	6.8	
Question 16 (deepness of sleep)	point	Active group (*n* = 33)	20.8	6.4	0.99	21.7	6.8	0.30	0.9	7.7	0.39
		Placebo group (*n* = 33)	20.7	7.5		20.1	7.1		−0.7	7.1	

The data are presented as the mean and standard deviation (SD). The baseline (Scr) values are evaluated using Student's *t*-test, the measured values are evaluated using Post-hoc comparisons with linear mixed model, with the baseline values utilized as covariates and time, group, group–time interaction, and subject as factors, and the changes are evaluated using Post-hoc comparisons with linear mixed model, with time, group, group–time interaction, and subject as factors.

*: $P < 0.05$ vs. the placebo group.

Table 4-2. OSA-MA (subgroup analysis of subjects aged 45 years and older in FAS)

	Unit		Scr			8wks			8wks−Scr		
			Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value
Sleepiness on rising	point	Active group (*n* = 18)	20.1	4.5	0.15	20.6	5.2	0.55	0.5	4.9	0.17
		Placebo group (*n* = 18)	22.7	6.1		20.7	5.7		−2.0	7.6	
Initiation and maintenance of sleep	point	Active group (*n* = 18)	19.7	5.1	0.29	21.0	5.2	0.15	1.3	4.1	0.07
		Placebo group (*n* = 18)	21.7	6.0		19.7	4.6		−2.0	5.8	
Frequent dreaming	point	Active group (*n* = 18)	26.9	4.0	0.07	24.5	5.5	0.47	−2.3	5.4	0.68
		Placebo group (*n* = 18)	23.6	6.2		22.1	5.3		−1.6	7.0	
Feeling refreshing	point	Active group (*n* = 18)	21.0	4.5	0.34	21.9	6.0	0.09	0.9	5.3	0.047*
		Placebo group (*n* = 18)	22.6	5.4		19.6	5.6		−3.0	7.2	
Sleep length	point	Active group (*n* = 18)	22.3	4.5	0.36	22.7	4.9	0.77	0.4	3.7	0.50
		Placebo group (*n* = 18)	23.9	6.0		23.1	5.2		−0.9	6.2	
question 1 (remaining fatigue)	point	Active group (*n* = 18)	20.9	5.3	0.83	21.3	5.3	0.12	0.4	6.3	0.18
		Placebo group (*n* = 18)	21.4	6.9		18.5	6.5		−2.9	9.0	
question 2 (concentration ability)	point	Active group (*n* = 18)	20.4	5.0	0.14	21.4	5.8	0.78	0.9	7.1	0.22
		Placebo group (*n* = 18)	23.9	8.5		21.8	6.1		−2.2	9.4	
question 3 (deepness of sleep)	point	Active group (*n* = 18)	16.9	5.8	0.09	19.3	6.8	0.30	2.4	6.5	0.06
		Placebo group (*n* = 18)	20.9	7.5		18.8	5.5		−2.1	8.5	
question 4 (relaxing)	point	Active group (*n* = 18)	20.6	4.8	0.54	21.8	4.7	0.20	1.2	5.8	0.69
		Placebo group (*n* = 18)	19.2	8.5		19.3	6.0		0.1	10.1	
question 5 (dullness)	point	Active group (*n* = 18)	19.2	4.7	0.22	20.8	6.9	0.05	1.6	7.0	0.021*
		Placebo group (*n* = 18)	21.3	5.6		18.0	5.6		−3.3	7.1	
question 6 (appetite)	point	Active group (*n* = 18)	25.9	8.0	0.28	23.7	8.9	0.98	−2.2	5.3	0.69
		Placebo group (*n* = 18)	28.7	7.5		25.6	7.7		−3.1	6.2	

	Unit		Scr			8wks			8wks−Scr		
			Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value
question 7 (dozing off)	point	Active group (*n* = 18)	20.7	6.3	0.29	22.6	5.6	0.31	1.8	5.4	0.14
		Placebo group (*n* = 18)	23.2	7.3		21.4	7.5		−1.7	8.0	
question 8 (clearness of thought)	point	Active group (*n* = 18)	19.7	6.5	0.11	20.1	6.6	0.51	0.4	6.3	0.14
		Placebo group (*n* = 18)	23.2	6.5		20.1	7.0		−3.2	8.8	
question 9 (nightmares)	point	Active group (*n* = 18)	27.5	4.6	0.31	26.2	4.3	0.32	−1.3	5.8	0.88
		Placebo group (*n* = 18)	25.7	6.0		24.1	5.5		−1.6	6.9	
question 10 (quality of sleep)	point	Active group (*n* = 18)	17.8	7.2	0.41	19.8	6.1	0.38	1.9	6.6	0.23
		Placebo group (*n* = 18)	19.8	7.2		18.8	6.5		−1.1	6.9	
question 11 (feelings)	point	Active group (*n* = 18)	23.0	5.3	0.24	23.6	6.7	0.26	0.6	5.0	0.12
		Placebo group (*n* = 18)	25.2	5.9		22.4	6.1		−2.8	8.4	
question 12 (dreaming)	point	Active group (*n* = 18)	26.2	5.0	0.040*	22.9	7.3	0.74	−3.3	6.9	0.46
		Placebo group (*n* = 18)	21.6	7.7		20.1	7.4		−1.5	8.3	
question 13 (waking at night)	point	Active group (*n* = 18)	21.8	6.4	0.79	21.7	6.6	0.19	−0.2	6.7	0.22
		Placebo group (*n* = 18)	22.4	6.2		19.3	5.1		−3.1	6.6	
question 14 (answering ability)	point	Active group (*n* = 18)	19.7	6.5	0.018*	19.2	6.3	0.85	−0.4	5.3	0.19
		Placebo group (*n* = 18)	24.6	5.4		21.7	6.9		−2.9	6.8	
question 15 (sleeping time)	point	Active group (*n* = 18)	18.7	5.2	0.83	21.7	4.2	0.47	3.0	4.2	0.46
		Placebo group (*n* = 18)	19.1	6.9		20.5	5.8		1.4	7.4	
question 16 (deepness of sleep)	point	Active group (*n* = 18)	21.1	6.9	0.66	21.6	6.2	0.40	0.5	6.1	0.33
		Placebo group (*n* = 18)	22.2	7.2		20.3	8.3		−1.8	7.2	

The data are presented as the mean and standard deviation (SD). The baseline (Scr) values are evaluated using Student's *t*-test, the measured values are evaluated using Post-hoc comparisons with linear mixed model, with the baseline values utilized as covariates and time, group, group–time interaction, and subject as factors, and the changes are evaluated using Post-hoc comparisons with linear mixed model, with time, group, group–time interaction, and subject as factors.

*: $P < 0.05$ vs. the placebo group.

VAS of fatigue: There were no significant differences between the groups (data not shown).

Safety assessment: The results of muscle hardness is shown in Appendices 3-1 to 3-3.

Although significant differences were observed between the groups in some of the test items, it was judged that the differences were not clinically meaningful because the measured values of the relevant items were within the reference values and the changes were not medically problematic. Therefore, no medically problematic changes were observed with the continued ingestion of the test food.

DISCUSSION

According to the prior *in vitro* study, curcumin has been reported to inhibit the expression of nuclear factor-kappa B (NF-κB), induced by the inflammatory cytokine tumor necrosis factor-α (TNF-α) [32]. NF-κB is a transcription factor and cell adhesion molecule that induces the onset of inflammation associated with the invasion of bacterial lipopolysaccharide and exogenous inflammatory substances [33] and the pathogenesis of autoimmune diseases [34]. NF-κB is activated by stimuli such as TNF-α, but in the absence of this stimuli, it forms a complex with the inhibitor protein, inhibitor of NF-κB (IκB) [34]. Once NF-κB is stimulated by TNF-α, IκB is phosphorylated, ubiquitinated, and degraded by IκB kinase [35]. This cascade reaction of NF-κB activation induces the transcription of cell adhesion molecules [34,35]. Inflammation is a biological defense mechanism against pathogens such as bacteria and is caused by the accumulation of leukocytes at the site of damage [3]. The pathogenesis of hepatic inflammatory diseases, such as alcoholic hepatitis and NASH, are also related to the accumulation of leukocytes [3]. Moreover, cell adhesion molecules are involved in the induction of leukocyte accumulation at the site of damage, and it is thought that suppressing the induction of leukocyte accumulation by cell adhesion molecules may reduce inflammation [36,37]. Since curcumin and its metabolites have anti-inflammatory effects [10,38], Theracurmin® may also suppress liver inflammation through the regulation of NF-κB expression. In fact, the cell counts of leukocyte, neutrophil, and lymphocyte in the active group was significantly lower than that in the placebo group, and a significant decrease in various leukocytes subtypes was observed at 8wks in this study. As mentioned above, hepatic inflammatory diseases are caused by the accumulation of leukocytes at the site of damage [3], and the leukocyte count in the blood also increases in conjunction with tissue damage [39]. Therefore, it is assumed that Theracurmin® exerted its anti-inflammatory effect in this study.

Excessive alcohol consumption is known to increase the levels of biomarkers of liver function, including the evaluation items of this study (ALT, AST, and γ-GT) [4]. In addition, it has been confirmed that the levels of these liver function markers were decreased by the intervention of health guidance to reduce alcohol consumption [40], and the presence or amount of alcohol consumption during the study period may have affected the results of this study. Thus, in this study, we conducted a PPS analysis that excluded the subjects who drank alcohol in amounts that increased the risk of lifestyle-related diseases (mean net alcohol intake of more than 40 g/day for men and more than 20 g/day for women) [27]. In the PPS analysis, the measured value and amount of change of ALT at 8wks were significantly lower in the active group than in the placebo group. This result means that the improvement in liver function was also observed in the subjects who did not drink

excessively. Chronic intake of alcohol is known to make acetaldehyde derived from ethanol bind to various proteins and membrane substances and denature them, resulting in fatty liver, hepatocellular death, inflammation, and decreased metabolism of substances [41]. Therefore, it can be inferred that the subjects who were excluded from the PPS analysis had reduced anti-inflammatory function in the liver. Aminotransferases, such as ALT and AST, are known to elevate in response to damage to cell membranes [42]. Particularly, the response of ALT is highly specific for liver disease [4]. Therefore, Theracurmin® is useful for maintaining and improving liver function in subjects who do not drink excessively and whose liver function is relatively good.

Curcumin is also expected to decrease fatigue and mental stress by suppressing TNF-α expression. It is known that TNF-α is highly expressed in the brains of rats subjected to chronic mild mental stress [43], and the blood TNF-α levels in humans is also reported to be increased by mental stress testing [44]. However, the *in vivo* study with rats reported that curcumin administration suppressed the expression of TNF-α in the brains of rats subjected to mild chronic mental stress [45]. It has been suggested that stress-induced inflammatory cytokines, such as TNF-α, activate the dorsal anterior cingulate cortex, a brain region activated by social stress, and induce negative affect[44]. In addition, overproduction of inflammatory cytokines is reported to induce the reactivation of herpesvirus, one of the fatigue-related biomarkers [46]. Therefore, suppression of TNF-α expression by curcumin intake is expected to reduce fatigue and mental stress.

In this study, a comparison of the amount of change in question 11 (feelings) of OSA-MA, showed significantly higher values in the active group than in the placebo group. In a subgroup analysis in FAS with subjects aged 45 years or older, defined by the MHLW as middle-aged and older persons in the Act on Stabilization of Employment of Elderly., fatigue recovery score and question 5 (dullness) were significantly higher in the active group than in the placebo group. In OSA-MA, question 5 (dullness) and question 11 (feelings) are components of Factor IV (fatigue recovery), and higher values indicate a better condition [23]. On the other hand, there was no significant difference between the groups in the VAS of fatigue. The reason for the inconsistent results between OSA-MA and the VAS of fatigue may be the difference in the evaluation timing.

It has been reported that there is a diurnal variation in fatigue [47]. The OSA-MA was answered immediately after waking up, whereas the VAS for fatigue was answered when the subjects came to the clinic for examination in this study. Subjective symptoms such as drowsiness and loss of energy are often reported mainly in the afternoon, reflecting circadian rhythms, but are less likely to be carried over to the next day [47]. However, dullness reflects diurnal or accumulative fatigue, and is thought to have greater diurnal variation than intraday variation [47]. In this study, dullness was reduced at the time of waking up, which is the time when the influence of diurnal variation is small, suggesting that the fatigue-reducing effect of Theracurmin® is effective against fatigue accumulated from the previous day. From the above, it is thought that fatigue and mental stress were reduced through the anti-inflammatory effect of curcumin, and subjective symptoms, which are partly responsible for the feeling of recovery from fatigue experienced upon waking, were improved.

It has been reported that blood levels of the inflammatory cytokines such as TNF-α, interleukin-1, and interleukin-6 increase with aging, and this is thought to be associated with the risk of developing age-related diseases and death [48]. The increase in the inflammatory cytokines is thought to be due to

cell debris accumulated with aging, toxic products of the oral and intestinal flora, and cellular senescence [49]. According to the epidemiological studies about the blood levels of inflammatory markers (CRP, TNF-α, and interleukin-6) in healthy subjects, all markers showed a remarkable increase in those over 40 to 50 years of age [50]. In the subgroup analysis of the subjects under 45 years of age in this study, the sleep length scores of OSA-MA at 8wks and 8wks−Scr were significantly higher in the active group than in the placebo group (Appendix 1). In the subgroup analysis of the subjects aged 45 years and older, the antifatigue effect of the test food was more pronounced, indicating that Theracurmin® has an antifatigue effect on middle-aged and elderly people, who are expected to have increased inflammatory cytokines.

CONCLUSION

In this study, we investigated the effects of Theracurmin® on liver function, fatigue, and sleep. There was a significant decrease in ALT, a highly specific marker for liver disease, in the subjects who did not drink alcohol excessively, indicating that Theracurmin® is useful for improving liver function. In addition, it was confirmed that the subjective symptoms, which play a part in recovery from fatigue upon waking, were significantly maintained, and the effect of Theracurmin® was more pronounced in persons aged 45 years and older, defined as “middle-aged or older” by the Ordinance of the MHLW in Act on Stabilization of Employment of Elderly Persons. Furthermore, the test food was safe under the conditions of this study.

List of abbreviations: ALT: alanine aminotransferase, AST: aspartate aminotransferase, γ-GT: γ-glutamyl transpeptidase, VAS: visual analog scale, OSA-MA: OSA sleep inventory MA version, ANCOVA: analysis of covariance, FAS: full analysis set, PPS: per protocol set, NF-κB: nuclear factor-kappa B, TNF-α: tumor necrosis factor-α, IκB: inhibitor protein, inhibitor of NF-κB

Competing Interests: The sponsor of this study, Theravalues Corporation, entrusted ORTHOMEDICO Inc. with conducting the study. Yoshitaka Kuwabara, Akiko Hirose, and Hyunjin Lee are members of Theravalues Corporation, and Daisuke Hashimoto and Shin-ichiro Iio are employees of ORTHOMEDICO Inc. Tsuyoshi Takara (MD), the principal investigator of this study, is a staff of Medical Corporation Seishinkai, Takara Clinic and monitored all the conditions of the subjects.

Authors’ contributions: Conceptualization, Yoshitaka Kuwabara, Akiko Hirose, Hyunjin Lee, Daisuke Hashimoto, Shin-ichiro Iio, and Tsuyoshi Takara; Methodology, Yoshitaka Kuwabara, Akiko Hirose, Hyunjin Lee, Daisuke Hashimoto, and Shin-ichiro Iio; Formal analysis, Yoshitaka Kuwabara, Akiko Hirose, Hyunjin Lee, Daisuke Hashimoto, Shin-ichiro Iio, and Tsuyoshi Takara; Investigation, Tsuyoshi Takara; Resources, Yoshitaka Kuwabara, Akiko Hirose, Hyunjin Lee, Daisuke Hashimoto, Shin-ichiro Iio, and Takahiro Yamada; Writing –original draft, Yoshitaka Kuwabara, Akiko Hirose, Hyunjin Lee, Daisuke Hashimoto, and Shin-ichiro Iio; Writing –review and editing, Yoshitaka Kuwabara, Akiko Hirose, Hyunjin Lee, Daisuke Hashimoto, Shin-ichiro Iio, and Tsuyoshi Takara; Supervision, Tsuyoshi Takara; Project administration, Tsuyoshi Takara

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