than 90 000/mm^3, or a baseline hemoglobin value less than 10 g/dL. Patients with a complication or anamnesis that could possibly influence the safety or efficacy of ME3738 were also excluded. Concomitant drugs or therapies that could possibly influence the safety and efficacy of ME3738, such as systemic antiviral drug and double-membrane filtration plasmapheresis, were also prohibited during the study period.

This study was conducted in accordance with Good Clinical Practice guidelines, conforming to the Declaration of Helsinki. Written informed consent was obtained from all patients before their enrollment in this study, which was conducted with the approval of the institutional review board of each study site.

Safety and efficacy assessments

Assessments were performed at baseline before starting the study; after 2, 4, 8 and 12 weeks of treatment; every 4 weeks thereafter; at EOT; and during the follow-up period. Assessments included inquiries, vital sign measurements, laboratory tests, adverse event surveys and HCV RNA determinations.

When any adverse event was noted, follow-up examinations were performed in the subject concerned and, if necessary, after study completion.

The amount of HCV RNA during the study period was determined by the TaqMan polymerase chain reaction (PCR) method.

At each determination time point, the disappearance of HCV RNA was judged when no signal was detected with the TaqMan-PCR method. Efficacy was assessed as the HCV disappearance rate after 12 weeks of treatment and at week 24 of the follow-up period. When "HCV RNA undetectability" was maintained until week 24 of the follow-up period, SVR was judged in the subject concerned.

The 16 subjects who participated in the extended treatment study (study ME3738-12) after receiving the treatment in this study were tabulated as subjects without SVR.

Mutations of the HCV core protein (amino acid [a.a.]70 and a.a.91), mutations of the IFN-sensitivity determining region (ISDR) and single nucleotide polymorphisms (SNP) of the *IL28B* gene (five sites: rs8103142, rs11881222, rs8099917, rs12980275 and rs12979860) were determined as background factors of the subjects.

Statistical analysis

Background factors were tabulated by each treatment group, and the intergroup bias was assessed using Student's *t*-test or one-way ANOVA for continuance data and Fisher's exact test for categorical data. A two-sided significance level of 5% was adopted.

In the efficacy evaluation, the rate of subjects with SVR was calculated for each treatment group, and the intergroup difference and the 95% confidence interval was determined. In the safety evaluation, adverse events and adverse reactions were tabulated for each treatment group to determine the incidence rate. The Medical Dictionary for Regulatory Activities (MedDRA) ver. 14.0 was used for tabulation of adverse events.

RESULTS

OF THE 135 subjects (male, 49; female, 86) included in the study, 46 were in the ME3738 50-mg/day group, 45 in the ME3738 200-mg/day group and 44 in the ME3738 800-mg/day group. The mean age was 57.6 years (range, 23–76) with elderly subjects over 60 years occupying more than half of the study population. As for sex, elderly women were dominant.

As per the protocol, 24 subjects in whom the viral amount did not decrease from the baseline level by at least 2 log IU/mL after 12 weeks of treatment were discontinued from the study. An additional three subjects were discontinued for other reasons, resulting in a total of 27 subjects being withdrawn from the study. As a result, study treatment was continued in 108 subjects after 12 weeks of treatment.

Of these 108 subjects, 16 subjects were discontinued from the study for various reasons, including occurrence of an adverse event. A total of 92 subjects completed the 48-week treatment. Among the subjects in whom HCV RNA was positive after 12 weeks of treatment and negative conversion was achieved with 36 weeks of treatment (LVR), 16 subjects from whom consent was obtained participated in the extended treatment study (study ME3738-12) and received 24 weeks of extended treatment in continuation from the 48-week treatment in this study.

Therefore, the follow-up observation was performed in 47 subjects who achieved viral disappearance after 48 weeks of treatment and did not participate in the extended treatment study, and SVR was judged in eight of those subjects after 24 weeks of follow-up observation (Fig. 1).

The major patient background factors showed no biases among the three treatment groups (ME3738 50-mg/day, ME3738 200-mg/day and ME3738 800-mg/day groups) (Table 1). SNP of the *IL28B* gene were determined in 126 subjects who provided consent for

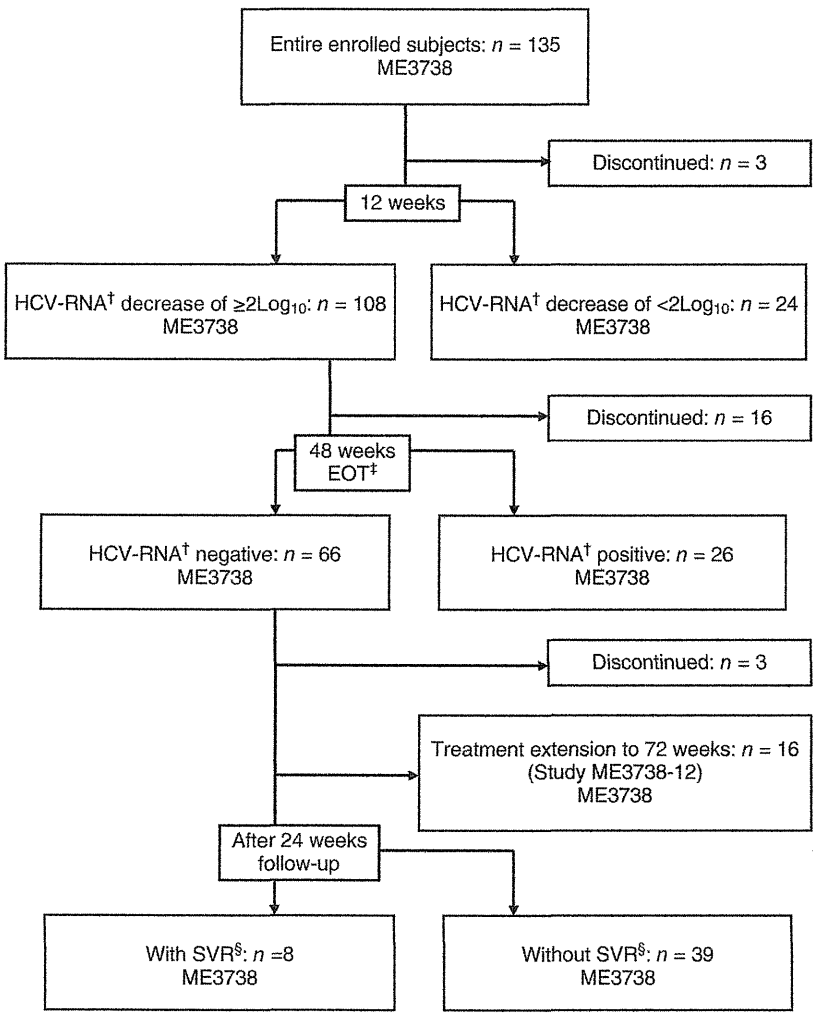

Figure 1 Subject disposition. †Hepatitis C virus. ‡End of treatment. §Sustained virological response.

the analysis. Among the 126 subjects, the result for rs12980275 was different from the results of the other four SNP in three subjects, whereas the results of all five SNP were coincident in the remaining 123 subjects. Even in the three subjects in whom the result for rs12980275 was different, the results for the other four SNP were coincident, allowing the coincident results of four SNP to be included in the tabulation.

The SNP of *IL28B* determined in this study were a major homo allele in 68.1% (92/126) of the subjects, a minor hetero allele in 24.4% (33/126) of the subjects and a minor homo allele in 0.7% (1/126) of the subjects. These rates were similar to the results previously reported in Japan.[11]

Efficacy

Table 2 shows the virological effects at each stage of this study. The viral disappearance rate indicating the proportion of subjects who achieved viral disappearance was 5.9% (8/135) after 4 weeks of treatment, 23.0% (31/135) after 12 weeks of treatment and 48.9% (66/135) at EOT. The rate of subjects with SVR was 5.9% (8/135) after 24 weeks of follow-up observation. The viral reactivation rate, meaning the proportion of subjects in whom viral regrowth was observed during the follow-up observation period from among the total subjects included in the follow-up observation, was 83.0% (39/47), and reactivation occurred within 12 weeks of

Table 1 Characteristics of study patients

		PEG IFN-α-2a§ + ME3738 50 mg/day ($n = 46$)	PEG IFN-α-2a§ + ME3738 200 mg/day ($n = 45$)	PEG IFN-α-2a§ + ME3738 800 mg/day ($n = 44$)	Total ($n = 135$)	Test result
Sex	Male	17 (37.0%)	16 (35.6%)	16 (36.4%)	49 (36.3%)	$P = 1.0000$ (Fisher's exact test)
	Female	29 (63.0%)	29 (64.4%)	28 (63.6%)	86 (63.7%)	
Age	Mean	56.5	59.8	56.6	57.6	$P = 0.3166$ (1-way ANOVA)
	Standard deviation	12.6	9.8	11.9	11.5	
	Median	61	61	60	60	
	Range	23–73	40–75	26–76	23–76	
Bodyweight	Mean	55.9	55.9	57.6	56.5	$P = 0.7033$ (1-way ANOVA)
	Standard deviation	12.1	11.3	9.8	11.1	
	Median	54.5	54.0	55.6	54.9	
	Range	34.9–88.6	39.2–82.5	43.0–84.0	34.9–88.6	
Latest liver biopsy result	F0–F2	21 (45.7%)	19 (42.2%)	14 (31.8%)	54 (40.0%)	$P = 0.4524$ (Fisher's exact test)
	F3	0 (0.0%)	1 (2.2%)	2 (4.5%)	3 (2.2%)	
	No test result	25 (54.3%)	25 (55.6%)	28 (63.6%)	78 (57.8%)	
Presence/absence of complication	Absence	2 (4.3%)	6 (13.3%)	5 (11.4%)	13 (9.6%)	$P = 0.3167$ (Fisher's exact test)
	Presence	44 (95.7%)	39 (86.7%)	39 (88.6%)	122 (90.4%)	
Presence/absence of diabetes	Absence	43 (93.5%)	45 (100.0%)	42 (95.5%)	130 (96.3%)	$P = 0.2843$ (Fisher's exact test)
	Presence	3 (6.5%)	0 (0.0%)	2 (4.5%)	5 (3.7%)	
Presence/absence of concomitant drug	Absence	0 (0.0%)	0 (0.0%)	1 (2.3%)	1 (0.7%)	$P = 0.3259$ (Fisher's exact test)
	Presence	46 (100.0%)	45 (100.0%)	43 (97.7%)	134 (99.3%)	
Presence/absence of concomitant therapy	Absence	29 (63.0%)	32 (71.1%)	35 (79.5%)	96 (71.1%)	$P = 0.2356$ (Fisher's exact test)
	Presence	17 (37.0%)	13 (28.9%)	9 (20.5%)	39 (28.9%)	
Baseline hemoglobin	Mean	13.9	14.2	14	14	$P = 0.6521$ (1-way ANOVA)
Pegasys dose change	No	26 (56.5%)	29 (64.4%)	25 (56.8%)	80 (59.3%)	$P = 0.8599$ (Fisher's exact test)
	Dose reduction	13 (28.3%)	12 (26.7%)	12 (27.3%)	37 (27.4%)	
	Dose withdrawal	7 (15.2%)	4 (8.9%)	7 (15.9%)	18 (13.3%)	
HCV RNA-1b† (NS5A)	0 or 1	45 (97.8%)	39 (86.7%)	40 (90.9%)	124 (91.9%)	$P = 0.1333$ (Fisher's exact test)
	Not less than 2	1 (2.2%)	6 (13.3%)	4 (9.1%)	11 (8.1%)	
HCV RNA-1b† IFN/RBV mutation at 70	Wild type	37 (80.4%)	26 (57.8%)	29 (65.9%)	92 (68.1%)	$P = 0.0611$ (Fisher's exact test)
	Mutant type/ competitive type	9 (19.6%)	19 (42.2%)	14 (31.8%)	42 (31.1%)	
	Other	0 (0.0%)	0 (0.0%)	1 (2.3%)	1 (0.7%)	
HCV RNA-1b† IFN/RBV‡ mutation at 91	Wild type	34 (73.9%)	23 (51.1%)	31 (70.5%)	88 (65.2%)	$P = 0.0529$ (Fisher's exact test)
	Mutant type/ competitive type	12 (26.1%)	22 (48.9%)	13 (29.5%)	47 (34.8%)	
Baseline HCV RNA†	Mean	6.7	6.6	6.7	6.6	$P = 0.4157$ (1-way ANOVA)
FibroIndex	Mean	1.43	1.5	1.41	1.45	$P = 0.5513$ (1-way ANOVA)
IL-28B	Not determined	5 (10.9%)	1 (2.2%)	3 (6.8%)	9 (6.7%)	$P = 0.8000$ (Fisher's exact test)
	Major homo allele	31 (67.4%)	32 (71.1%)	29 (65.9%)	92 (68.1%)	
	Minor hetero allele	9 (19.6%)	12 (26.7%)	12 (27.3%)	33 (24.4%)	
	Minor homo allele	1 (2.2%)	0 (0.0%)	0 (0.0%)	1 (0.7%)	

†Hepatitis C virus.
‡Interferon/ribavirin.
§Pegylated interferon alpha-2a.

Table 2 Undetectable HCV RNA during the treatment period

No. of subjects (%)		Treatment period						
		4 weeks	8 weeks	12 weeks	24 weeks	48 weeks (EOT†)	Follow up‡ (12 weeks)	Follow up‡ (24 weeks)
PEG IFN-α-2a§ + ME3738 50 mg/day	$n = 46$	2	8	12	23	23	3	3
		4.3%	17.4%	26.1%	50.0%	50.0%	6.5%	6.5%
PEG IFN-α-2a§ + ME3738 200 mg/day	$n = 45$	3	4	12	20	26	5	3
		6.7%	8.9%	26.7%	44.4%	57.8%	11.1%	6.7%
PEG IFN-α-2a§ + ME3738 800 mg/day	$n = 44$	3	5	7	12	17	3	2
		6.8%	11.4%	15.9%	27.3%	38.6%	6.8%	4.5%
Total	$n = 135$	8	17	31	55	66	11	8
		5.9%	12.6%	23.0%	40.7%	48.9%	8.1%	5.9%

†End of treatment.
‡12/24 weeks after EOT.
§Pegylated interferon-α-2a.

follow-up observation in most of the subjects who showed viral reactivation.

Table 3 shows the patient background factors which might, and are considered to, have influenced the SVR in this study. With respect to sex, the effect was higher in men than in women, and with respect to age, the effect was lower in the elderly (≥60 years) than in younger subjects (<60 years). Thus, the effect was the lowest in elderly female subjects. There were no definite differences in FibroIndex showing the grade of liver fibrosis. In terms of ISDR, which is a predictive viral factor for IFN therapy in patients infected with HCV genotype 1b, the effect was high in mutants (no. of mutations,: ≥2), but no clear results were obtained in mutations of Core 70 and Core 91. Finally, in terms of *IL28B* SNP, SVR was only seen with the major homo allele.

Treatment with ME3738 showed no clear influence on ALT levels in this study (no relevant data are shown).

Safety

At least one adverse event was noted to 134 subjects among 135 subjects, excluding one subject in whom the study was discontinued after 5 weeks of treatment. Table 4 shows the adverse events noted with an incidence of at least 20% in the entire study population.

Frequently observed adverse events were fever, malaise, headache, nasopharyngitis, pruritus, retinopathy and diarrhea. Frequently observed abnormal laboratory findings were decreased white blood cell count, decreased platelet count, decreased neutrophil count, increased hyaluronic acid, decreased hemoglobin, decreased hematocrit and decreased red blood cell count.

In this study, decreased hemoglobin was observed in 45.9% (62/135) of subjects, and the hemoglobin level decreased to less than 10 g/dL in the treatment period in 19.3% (26/135) of subjects. Figure 2 shows the time-course of changes in the hemoglobin level in each ME3738 treatment group.

DISCUSSION

IN THIS STUDY conducted in patients with naïve HCV, 135 subjects were administrated ME3738 and 27 subjects were withdrawn from this study by week 12 for various reasons, including applicability to the study discontinuation criteria specified in the protocol. As a result, study treatment was continued in 108 subjects after 12 weeks of treatment. Among these, 16 subjects discontinued the study for reasons such as occurrence of an adverse event. There were 92 subjects who completed

Table 3 Comparison of clinical characteristics and viral types between subjects with and without sustained virological response

	Virological response	
	Subjects with SVR†, *n* (%)	Subjects without SVR†, *n* (%)
SVR†		
Entire subjects	8/135 (5.9%)	127/135 (94.1%)
By sex and age		
Men	5/49 (10.2%)	44/49 (89.8%)
Age, <60 years	4/23 (17.4%)	19/23 (82.6%)
Age, ≥60 years	1/26 (3.8%)	25/26 (96.2%)
Women	3/86 (3.5%)	83/86 (96.5%)
Age, <60 years	3/36 (8.3%)	33/36 (91.7%)
Age, ≥60 years	0/50 (0%)	50/50 (100%)
FibroIndex		
>1.25	5/95 (5.3%)	90/95 (94.7%)
≤1.25	3/40 (7.5%)	37/40 (92.5%)
ISDR‡		
Wild (0–1)	5/124 (4.0%)	119/124 (96.0%)
Mutant (>2)	3/11 (27.3%)	8/11 (72.7%)
Core region amino acid substitution site		
Wild	5/92 (5.4%)	87/92 (94.6%)
70-mutant	3/43 (7.0%)	40/43 (93.0%)
Wild	2/88 (2.3%)	86/88 (97.7%)
91-mutant	6/47 (12.8%)	41/47 (87.2%)
Genotyping of *IL-28B* SNP§		
Major homo allele	8/92 (8.7%)	84/92 (91.3%)
Minor hetero/homo allele	0/34 (0%)	34/34 (100%)

†Sustained virological response.
‡Interferon sensitivity determining region.
§Single nucleotide polymorphism.

the 48-week treatment. As compared with the standard combination therapy of PEG IFN-α-2b and RBV, it appeared that the subject withdrawal rate was lower and the treatment completion rate was higher in the current study.

An antiviral effect was seen in 48.9% of subjects after 48 weeks of treatment, but SVR was judged in only 5.9% of subjects at the end of the follow-up observation period. On the other hand, in a clinical study of combination therapy with PEG IFN-α-2a plus RBV conducted in Japan in patients with naïve chronic hepatitis C (HCV genotype 1b, high viral load), the viral disappearance rate was 86.9% (86/99) and SVR was judged in 59.4% (57/96) of subjects at EOT.[12] In the combination therapy with ME3738 plus PEG IFN-α-2a, the proportion of subjects with SVR was markedly lower than that of subjects who achieved viral disappearance after 12 or 48 weeks of treatment, suggesting that suppression of viral reactivation is weaker with the combination therapy that includes ME3738 than with the combination therapy that includes RBV.

In addition, there were no differences in SVR among the three different ME3738 doses.

In terms of the influence of ME3738 treatment on ALT levels, no consistent tendency could be found in the combination therapy used in this study.

In the safety evaluation, most adverse events noted in this study were comparable in severity and frequency with the events frequently noted with PEG IFN-α-2a monotherapy,[13] and no new adverse events were noted with the ME3738 combination therapy, apart from the two events described below. This suggests that the adverse events noted with ME3738 plus PEG IFN-α-2a combination therapy were not substantially different in severity and frequency from the adverse events noted with PEG IFN-α-2a monotherapy, and that the safety of ME3738 combination therapy is high.

Increased hyaluronic acid and increased blood immunoglobulin G were adverse events that were not frequently seen with PEG IFN-α-2a monotherapy, but were frequently seen in this study. Because these parameters are seldom determined in a time-course manner in

Table 4 Most common adverse events

n (%)	PEG IFN-α-2a† + ME3738 50 mg/day (*n* = 46)	PEG IFN-α-2a† + ME3738 200 mg/day (*n* = 45)	PEG IFN-α-2a† + ME3738 800 mg/day (*n* = 44)	Total (*n* = 135)
Eye disorders				
Retinopathy	14 (30.4%)	15 (33.3%)	13 (29.5%)	42 (31.1%)
Gastrointestinal disorders				
Diarrhea	19 (41.3%)	14 (31.1%)	9 (20.5%)	42 (31.1%)
General disorders and administration site conditions				
Malaise	21 (45.7%)	18 (40.0%)	18 (40.9%)	57 (42.2%)
Fever	27 (58.7%)	22 (48.9%)	31 (70.5%)	80 (59.3%)
Infections and infestations				
Nasopharyngitis	16 (34.8%)	15 (33.3%)	12 (27.3%)	43 (31.9%)
Metabolism and nutrition disorders				
Decreased appetite	8 (17.4%)	8 (17.8%)	13 (29.5%)	29 (21.5%)
Musculoskeletal and connective tissue disorders				
Arthralgia	14 (30.4%)	15 (33.3%)	11 (25.0%)	40 (29.6%)
Nervous system disorders				
Headache	17 (37.0%)	15 (33.3%)	16 (36.4%)	48 (35.6%)
Skin and subcutaneous tissue disorders				
Alopecia	13 (28.3%)	14 (31.1%)	8 (18.2%)	35 (25.9%)
Pruritus	11 (23.9%)	15 (33.3%)	17 (38.6%)	43 (31.9%)
Investigations				
White blood cell count decreased	40 (87.0%)	40 (88.9%)	37 (84.1%)	117 (86.7%)
Red blood cell count decreased	20 (43.5%)	14 (31.1%)	21 (47.7%)	55 (40.7%)
Hemoglobin decreased	24 (52.2%)	15 (33.3%)	23 (52.3%)	62 (45.9%)
Hematocrit decreased	18 (39.1%)	17 (7.8%)	21 (47.7%)	56 (41.5%)
Platelet count decreased	35 (76.1%)	39 (86.7%)	36 (81.8%)	110 (81.5%)
Neutrophil count decreased	32 (69.6%)	37 (82.2%)	38 (86.4%)	107 (79.3%)
Aspartate aminotransferase increased	11 (23.9%)	11 (24.4%)	10 (22.7%)	32 (23.7%)
Blood triglycerides increased	11 (23.9%)	8 (17.8%)	8 (18.2%)	27 (20.0%)
Hyaluronic acid increased	23 (50.0%)	21 (46.7%)	19 (43.2%)	63 (46.7%)
Blood immunoglobulin G increased	10 (21.7%)	8 (17.8%)	9 (20.5%)	27 (20.0%)
Total	46 (100%)	45 (100%)	43 (97.7%)	134 (99.3%)

†Pegylated interferon-α-2a.

regular clinical practice, these two events have not been previously reported, and it was unclear whether these events were caused by ME3738 plus PEG IFN-α-2a combination therapy or by the primary disease itself. Furthermore, in subjects who developed these two events, no particular symptoms or findings were reported, suggesting that these two events may not be clinically problematic.

In a double-blind clinical study conducted in Japan comparing PEG IFN-α-2a plus RBV combination therapy and PEG IFN-α-2a plus placebo (48-week treatment) combination therapy, the reported incidence of decreased hemoglobin was 89.9% and 49.5%, respectively,[12] and the incidence of decreased hemoglobin noted in the present study was 45.9%. Considering the severity of decreased hemoglobin, the proportion of subjects in whom the hemoglobin level decreased to less than 10 g/dL and that resulted in dose reduction or withdrawal of RBV was 33.0% in the group receiving the PEG IFN-α-2a plus RBV combination and 4.0% in the group receiving the PEG IFN-α-2a plus placebo combination.[12] In the present study, the proportion of subjects in whom the hemoglobin level decreased to less than 10 g/dL during the study period was 19.3%.

In terms of the incidence of decreased hemoglobin, when patient background factors were compared

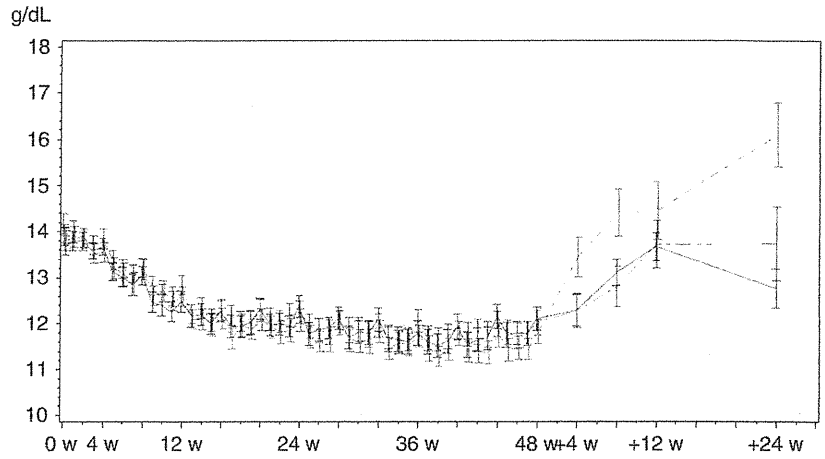

Figure 2 Time-course changes in hemoglobin levels. ----, ME3738 50 mg/day; ····, ME3738 200 mg/day; ——, ME3738 800 mg/day.

between the present study and the above double-blind study (the RBV co-administration study), the mean age was higher in the present study by approximately 5 years, and the male : female ratio was 4:6 in the present study, but 7:3 in the RBV co-administration study.[12] Furthermore, the mean baseline hemoglobin level was 14.0 g/dL in the present study, but 14.76 g/dL in the RBV co-administration study.[13] The incidence of decreased hemoglobin was lower in the present study than in the RBV co-administration study despite less favorable patient background factors in the present study.

As described, ME3738 was found to have a good safety profile and showed inflammatory suppression effects in the liver. In addition, Ogasawara *et al.* reported that ME3738 showed antiproliferative effects on liver cancer cells in an *in vitro* and *in vivo* liver tumor nude mouse model using hepatocellular carcinoma cell lines.[14] These findings suggest that long-term co-administration of ME3738 (which does not cause severe hemoglobin decrease) with PEG IFN is a therapeutic option anticipated to suppress inflammation in the liver, decrease the amount of HCV RNA, suppress proliferation of HCV, and suppress the onset of liver cancer in patients with chronic hepatitis C in whom the standard combination therapy with PEG IFN and RBV cannot be used because of a decrease in hemoglobin levels or because the patient is elderly.

ME3738 was concurrently used with PEG IFN-α-2a treatment; however, a clear additional effect on SVR was not confirmed in this trial.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Bofutsushosan, a Japanese herbal (Kampo) medicine, attenuates progression of nonalcoholic steatohepatitis in mice

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Abstract
Background Obesity-induced liver disease (nonalcoholic fatty liver disease, NAFLD) is now the commonest cause of chronic liver disease in affluent nations. There are presently no proven treatments for NAFLD or its more severe stage, nonalcoholic steatohepatitis (NASH). Bofutsushosan (BTS), a Japanese herbal (Kampo) medicine, long used as an anti-obesity medicine in Japan and other Asian countries, has been shown to reduce body weight and improve insulin resistance (IR) and hepatic steatosis. The precise mechanism of action of BTS, however, remains unclear. To evaluate the ability of BTS to prevent the development of NASH, and determine the mediators and pathways involved.
Methods C57BL/6 mice were injected intra-peritoneally with gold-thioglucose and fed a high-fat diet (HF) or HF diet admixed with either 2 or 5 % BTS for 12 weeks. The effectiveness of BTS in attenuating features of NASH and the mechanisms through which BTS attenuated NASH were then assayed through an assessment of the anthropometric, radiological, biochemical and histological parameters.
Results BTS attenuated the progression of NASH through induction of adiponectin and its receptors along with an induction of PPAR-α and PPAR-γ, decreased expression of SREBP-1c, increased hepatic fatty acid oxidation and increased hepatic export of triglycerides. BTS moreover, reduced IR through phosphorylation of the protein kinase, Akt.
Conclusions BTS through induction of adiponectin signaling and Akt attenuated development of NASH. Identification of the active entity in BTS should allow development of novel treatments for NASH.

Keywords NAFLD · Adiponectin · Bofutsushosan · Kampo medicine

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
GTG	Gold-thioglucose
HF	High-fat-diet
BTS	Bofutsushosan
IR	Insulin resistance
IGT	Impaired glucose tolerance
GTT	Glucose tolerance test

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ITT	Insulin tolerance test
QUICKI	Quantitative insulin sensitivity check index
MCAD	Medium-chain acyl-CoA dehydrogenase
TIMP	Tissue inhibitor of metalloproteinases
TGF	Transforming growth factor
MTP	Microsomal triglyceride transfer protein
AMPK	AMP-activated protein kinase

Introduction

Obesity-induced liver disease (nonalcoholic fatty liver disease, NAFLD), is now the commonest cause of chronic liver disease in affluent nations. The disease comprises obesity and insulin resistance (IR), with a consequent histopathological spectrum of hepatosteatosis, steatohepatitis (nonalcoholic steatohepatitis, NASH), cirrhosis and possible hepatocellular cancer [1–3]. There are presently no proven treatments for NAFLD or its more severe stage NASH. Bofutsushosan (BTS), a Japanese herbal (Kampo) medicine, long used as an anti-obesity medicine in Japan and other Asian countries [4] has recently been shown in obese Japanese women, to reduce body weight and improve IR [4]. In addition, BTS in experimental animals prevented adipogenesis [5], reduced weight, suppressed visceral and subcutaneous fat accumulation and in parallel decreased plasma glucose, triglycerides (TG), insulin, tumor necrosis factor-alpha [6], and hepatic steatosis induced by high-fat diet feeding [7, 8]. The mechanism of action of BTS however, is not known. Our aim here was to evaluate the ability of BTS to prevent the development of NASH in a recently described murine model involving administration of gold-thioglucose (GTG) and high-fat feeding to induce NASH [9], and to determine the mediators and pathways involved.

Materials and methods

Animal preparation

All procedures conformed to our institutions' guidelines for the care and use of animals in Kochi Medical School. Four-week-old male C57BL/6 mice were purchased from CLEA Japan Inc. All animals were housed for 12 weeks on a 12 h light/12 h dark cycle, with food and water freely available. Mice were fed high-fat-diet (HF, 640 kcal/100 g, F2HFD2, Oriental Yeast, Tokyo, Japan) or HF admixed with 2 or 5 % BTS (TJ-62, Tsumura & Co., Tokyo, Japan). All groups were fed standard chow (SC) for the first week and then continued on their respective group diets for remainder of the protocol.

Three experimental groups were studied: (1) intra-peritoneal administered GTG (2 mg/g of body weight, Sigma-Aldrich, St. Louis, MO, USA) and then SC for 1 week followed by HF diet for 11 weeks (GTG + HF) [9]; (2 and 3) intra-peritoneal GTG, SC for 1 week followed by HF diet admixed with either 2 %BTS or 5 % BTS for further 11 weeks (2 %BTS or 5 % BTS). At the end of the treatment period, all animals were fasted overnight, anesthetized with pentobarbital sodium intraperitoneally (25–50 mg/kg of body weight, Nembutal; Abbott Laboratories, Abbott Park, IL, USA). Blood and liver samples were harvested. Livers were fixed in 10 % formalin, or snap frozen in liquid nitrogen and stored at −80 °C, for later analyses.

CT scan analysis for body fat composition

The extent of adiposity in each experimental group was assayed by CT scanning (La Theta, ALOKA, Tokyo, Japan) under isoflurane (2 % v/v) anesthesia as described previously [9]. Animal were scanned at 2-mm intervals from the diaphragm to the pelvis, and visceral fat and subcutaneous fat volumes quantified with La Theta software (version 1.00) [9–11].

Histopathological examination

Five-micrometer sections of formalin-fixed/paraffin-embedded livers were processed for haematoxylin and eosin (H&E). Oil Red-O staining of intracellular neutral lipids was performed according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). For estimation of extent of hepatic steatosis, the areas of digital photomicrographs were quantified with a computerized image analysis system (macintosh MacSCOPE version 2.591) as described previously [9, 12]. Degree of oxidative stress was determined by staining and quantification with anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) and anti-4-hydroxy-2-nonenal (4-HNE) as previously described [9, 12, 13].

Glucose tolerance test (GTT), insulin tolerance test (ITT) and QUICKI

At 12 weeks, a glucose tolerance test (GTT) ($n = 6$) and an insulin tolerance test (ITT) ($n = 6$) were performed. For GTT, mice were fasted for 18 h, and then intra-peritoneally loaded with 20 % glucose at a dose of 1.0 g/kg body weight. For ITT, mice were fasted for 6 h, and then intra-peritoneally challenged with human insulin at 1.0 U/kg body weight [14, 15]. With both GTT and ITT blood

samples from the orbital sinus were taken at times 0, 30, 45, 60 and 120 min and plasma glucose concentrations measured using an automatic blood glucose meter (Glutest; Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan). Plasma insulin level was measured by Ultrasensitive Mouse Insulin ELISA kit (Mercodia AB, Uppsala, Sweden) according to the manufacture's protocol. The quantitative insulin sensitivity check index (QUICKI), as a measure of IR, was calculated from the fasting insulin and glucose levels.

Measurement of plasma adiponectin

Plasma adiponectin levels were measured by Mouse Adiponectin/Acrp30 (R&D Systems, Minneapolis, MN, USA) according to the manufacture's instructions.

Laboratory evaluation

Asparate aminotransferase (AST), alanine aminotransferase (ALT) and TG were measured by an autoanalyzer (BM6010; JEOL Ltd., Tokyo, Japan).

Real-time RT-PCR for quantitative assessment of mRNA expression

Total RNA was extracted using trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacture's protocol. RNA extracts were reverse-transcribed with random hexamers and avian myeloblastosis virus reverse transcriptase using a commercial kit (Takara, Kyoto, Japan). Real time RT-PCR were performed for quantitative assessment of mRNA expression on an ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Probes and primers for TNF-α, PPAR-α, PPAR-γ, MTP, DGAT2, Cyp2E1, adiponectin receptor 1/receptor 2 (AdipoR1/R2) were all purchased from Applied Biosystems. Relative expression of target gene mRNA was normalized to the amount of GAPDH mRNA.

Western blot analysis

For in vivo analysis of phosphorylated Akt and total Akt, mice were fasted for 18 h, injected intra-peritoneally with human insulin (10 U/kg) or control, and sacrificed 4 min later. Livers were snap frozen in liquid nitrogen. Liver total protein was analyzed by western blot with a polyclonal antibody to phosphorylated Akt/Akt and phosphorylated AMPK/AMPK (Cell Signaling Technology, Inc., Danvers, MA, USA) as described [16]. For analysis of SREBP-1c, mice were fasted for 18 h, sacrificed, and livers dissected and homogenized to prepare cell nuclear extracts which were then analyzed by western blotting with anti-SREBP-1c antibody (Santa Cruz Biotechnology, Inc., California, CA, USA) as described [17]. For analysis of 4-HNE, liver total protein was analyzed by western blot with a monoclonal anti-4-HNE antibody (Japan Institute for the Control of Aging, Shizuoka, Japan).

Statistics

Data are shown as mean $\pm$ SD. A univariate analysis was conducted with the Mann–Whitney U test to determine significance between groups. Qualitative data were compared using Fisher's exact test. Statistical significance was accepted at $p < 0.05$. All analyses were performed using Stat View software (SAS Institute, Cary, NC, USA).

Results

BTS treatment reduces GTG + HF induced obesity and steatohepatitis

Mice administered GTG and then fed a HF diet (GTG + HF), had a comprehensive histological and dysmetabolic phenotype resembling human NASH as reported recently [9]. To then evaluate the effectiveness of BTS in the GTG + HF model, we studied the anthropometric, radiological, biochemical and histological parameters as detailed above in the presence or absence of BTS 2 or 5 % admixed with the HF component of the GTG + HF. Administration of BTS attenuated weight gain in a dose dependent manner (Fig. 1a). Unexpectedly, GTG + HF induced increase in the volume of the visceral and subcutaneous fat were not markedly attenuated by treatment with BTS (Fig. 1b). However, hepatic steatosis on H&E or Oil Red-O staining in 12 weeks was attenuated in a dose dependent manner by BTS treatment (Figs. 2a, 3a). Additionally, BTS treatment also attenuated GTG + HF induced hepatomegaly in a dose-dependent manner (Fig. 2b).

GTG + HF mice livers showed steatohepatitis with marked steatosis and inflammation, hepatocyte ballooning and Mallory–Denk bodies as described previously (Fig. 3a) [9]. BTS treatment attenuated hepatic steatosis and hepatic inflammation (Fig. 3a), and inhibited hepatocyte ballooning and Mallory–Denk bodies. In parallel, oxidative stress makers, 8-OHdG (Fig. 3b) and 4-HNE (supplemental figure), were remarkably reduced by BTS, as was the expression of TNF-α (Fig. 3b). BTS treatment moreover, attenuated GTG + HF induced elevation of transaminases (Table 1).

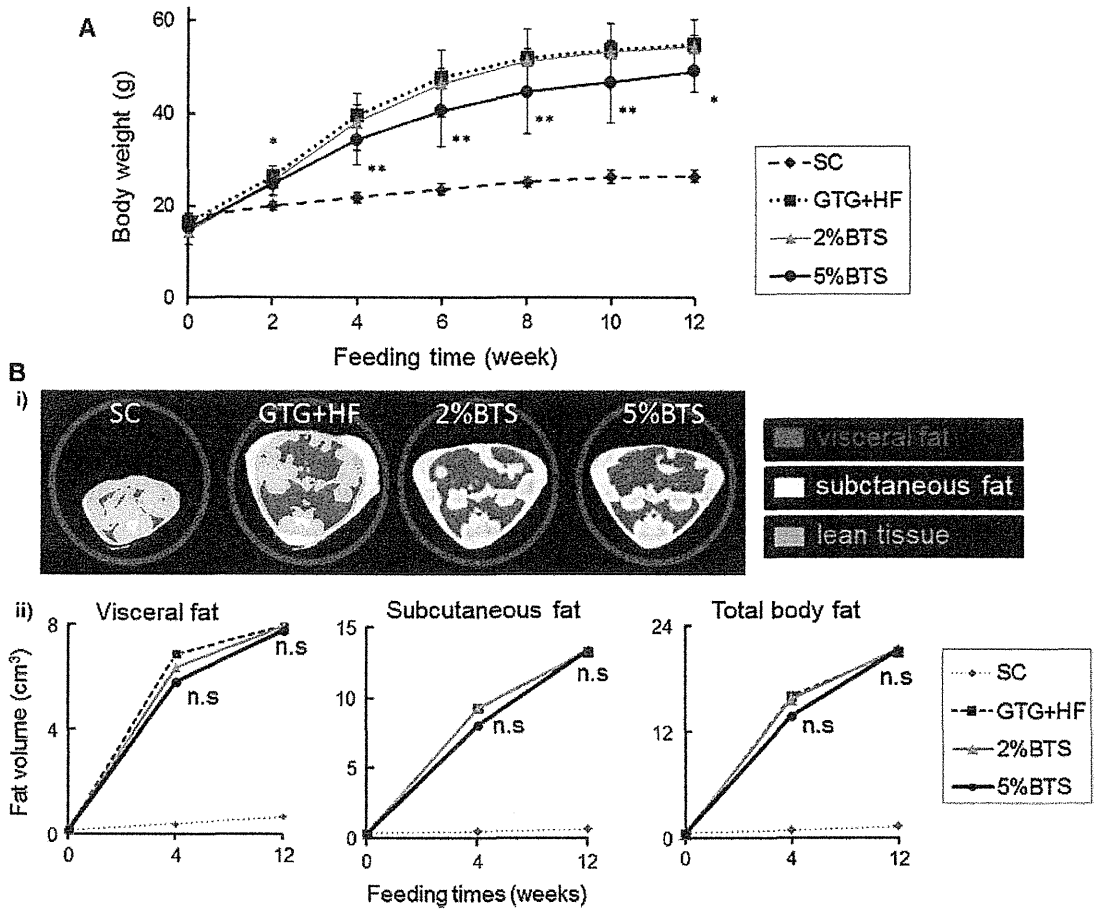

Fig. 1 Effectiveness of BTS for obesity and adiposity. **a** BTS dose-dependently attenuated weight gain in GTG + HF mice. $^{**}p < 0.01$, $^{*}p < 0.05$ vs GTG + HF, $n = 6$. *SC* standard chow fed mice, *GTG + HF* GTG treated and HF fed mice, *2 %BTS* 2 %BTS + GTG + HF fed mice, *5 %BTS* 5 %BTS + GTG + HF fed mice. **b** Anthropometry and evaluation of subcutaneous fat and visceral fat volumes by abdominal CT *i* representative photomicrographs of abdominal CT scan of SC, GTG + HF, 2 %BTS and 5 %BTS mice shown at 12 weeks. *Yellow* subcutaneous fat, *purple* visceral fat, *blue* lean tissue. *ii* Time course of increase of volume of subcutaneous and visceral fat. Neither subcutaneous fat nor visceral fat was significantly attenuated by treatment with BTS; 2 %BTS, 5 %BTS vs GTG + HF, p = ns, $n = 6$

BTS inhibition of hepatic lipid metabolism occurs through induction of adiponectin signaling

We examined plasma adiponectin levels and liver expression of the adiponectin receptors (AdipoR1 and AdipoR2), an important anti-inflammatory cytokine and receptors [18–20], because both plasma adiponectin levels and liver expression of the adiponectin receptors were decreased in GTG + HF mice as described previously [9]. Plasma adiponectin level and hepatic expression of AdipoR1, R2 were significantly enhanced by BTS (Fig. 4a). We next investigated the pathways of suppression of hepatic lipid metabolism by adiponectin in the presence of BTS. The expression of SREBP-1c was decreased dose dependently by BTS (Fig. 4b). The phosphorylation of AMPK (P-AMPK/AMPK) was here increased by BTS treatment (Fig. 4c) in parallel with activation of AdipoR1 signaling (Fig. 4a). In addition, expression of PPAR-γ, an activator of AMPK, was increased by BTS treatment (Fig. 4d).

Attenuation of hepatic steatosis with BTS treatment involves activation of fatty acid oxidation

Additionally, the expression of PPAR-α and its target genes, MCAD, involved in mitochondrial β-oxidation and CYP2E-1 involved in microsomal ω-oxidation [21, 22] were increased by treatment with BTS (Fig. 4e–g).

BTS promotes hepatic lipid export

We next determined if enhanced secretion of TG from the liver could contribute to the attenuation of hepatic steatosis by BTS treatment. The expression of microsomal triglyceride transfer protein (MTP) known to play a central role in

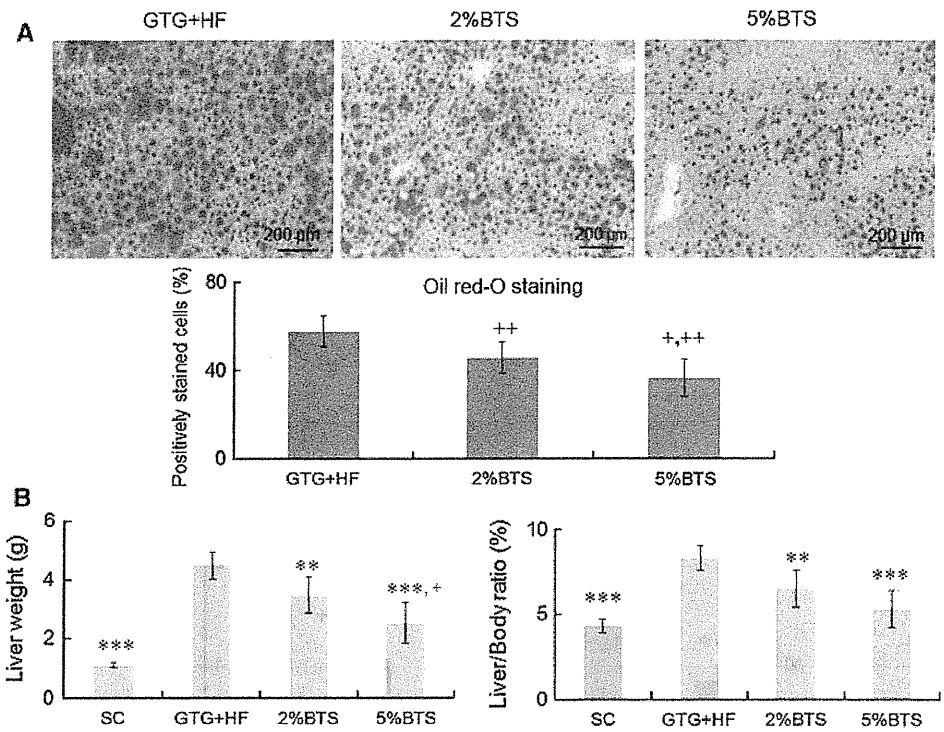

Fig. 2 BTS reduces hepatomegaly and hepatic steatosis. **a** Oil red-O staining and image analysis of livers: Oil red-O staining showed that BTS attenuated hepatic steatosis in GTG + HF fed mice. In addition, image analysis for Oil red-O staining of liver sections confirmed that BTS treatment attenuated hepatic steatosis. *Plus symbol* $p < 0.0001$ vs 2 %BTS, *double plus symbol* $p < 0.00001$ vs GTG + HF, $n = 6$. **b** Liver weight and liver/body weight ratio (liver/body): the increase of both liver weight and liver/body ratio was significantly attenuated by BTS treatment in a dose dependent manner. *Triple asterisk* $p < 0.001$, *double asterisk* $p < 0.01$ vs GTG + HF, *plus symbol* $p<0.05$ vs 2 %BTS, $n = 6$

lipoprotein assembly [23] was increased with BTS treatment (Fig. 4h). Interestingly, expression of DGAT2 reported to be involved in the conversion of free fatty acids into TG in the liver [24] was also increased in the BTS treated groups (Fig. 4i).

BTS reduces glucose intolerance and insulin resistance through induction of Akt

Since IR is regarded as a central pathogenic feature of NAFLD [25], we now investigated the effect of BTS treatment on IR. Fasting plasma glucose and insulin levels were markedly reduced in a dose dependent manner by BTS treatment, and QUICKI as an index of IR was also increased by BTS (Fig. 5a). We next evaluated the attenuation of glucose intolerance and IR using the GTT and the ITT in the presence of BTS. GTT revealed that treatment with BTS remarkably attenuated severe glucose intolerance induced by GTG + HF, and ITT showed that treatment with BTS attenuated IR induced by GTG + HF (Fig. 5b). The reduction of IR by BTS involved its promotion of the phosphorylation of Akt (Fig. 5c) an important factor in glucose metabolism [26].

Discussion

The public health importance of NAFLD [1, 2] and the unavailability of proven and effective therapies drive the search for a greater understanding of its pathophysiology and novel therapeutic pathways. In this study, we have clarified the mechanisms through which BTS attenuates NASH based on a novel animal model of NASH [9]. BTS attenuated the GTG + HF induced increases in body and liver weight, serum transaminases, hepatic steatosis, degree of oxidative stress and TNF-α expression (Figs. 1, 2, 3; Table 1) without reducing intake volume of diets (data not shown). However, in contrast to previous reports [6], we did not demonstrate statistically remarkable reduction by BTS, in GTG + HF mice, of induced increases in visceral or subcutaneous fat (Fig. 1b), perhaps because the volume of these fats in our mice were much larger than previously reported [6]. Additionally, the reduction of body weight might mainly be through the reduction of fat accumulation in muscles, since it has been reported that the degree of hepatosteatosis is well correlated with the degree of fat accumulation in muscles [27].

To examine the mechanisms though which BTS attenuated hepatic steatosis, we firstly evaluated the effect of BTS on adiponectin, thought to be a central adipokine in the pathogenesis for NASH [18, 19]. The expression of AdipoR1 and AdipoR2 in the livers was increased in a dose dependent manner by BTS (Fig. 4a), indicating that BTS could have PPAR-α agonist like effect, since PPAR-α agonists are known to increase expression of AdipoR1 and AdipoR2 [28]. Interestingly, plasma adiponectin levels were also remarkably induced by BTS treatment (Fig. 4a) even though neither visceral nor subcutaneous fat were decreased. These data indicated that BTS through putative

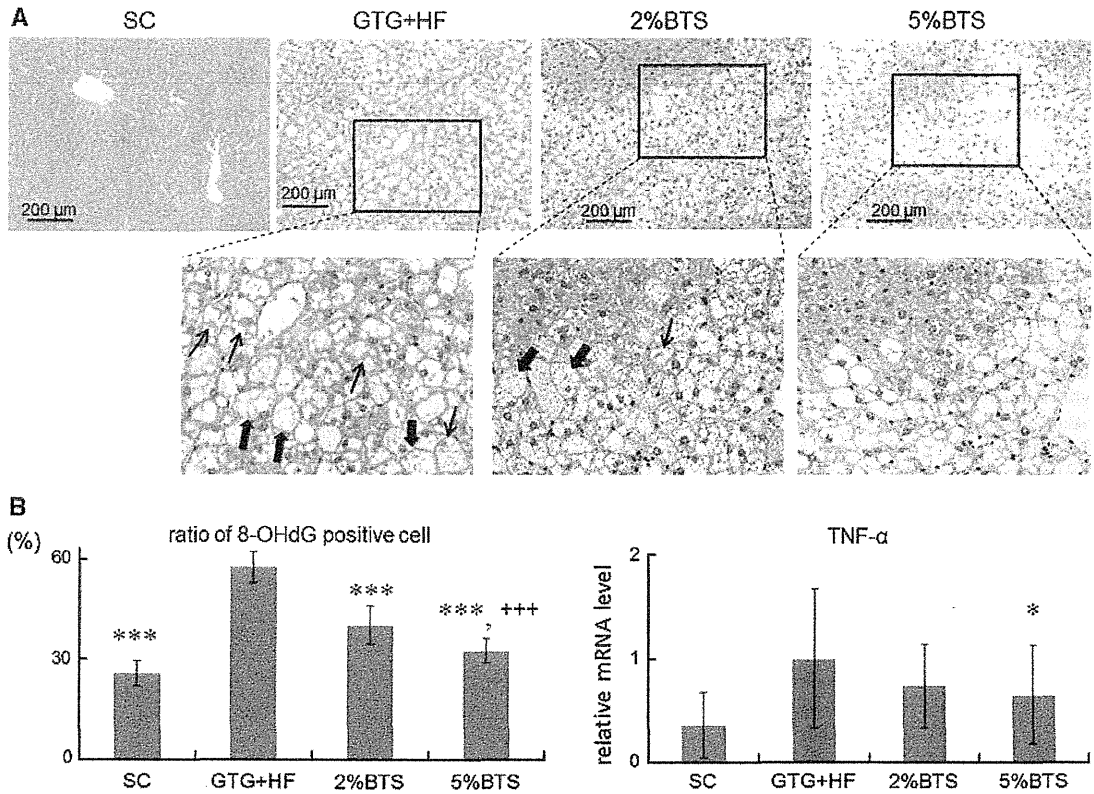

Fig. 3 BTS treatment reduces hepatic inflammation and oxidative stress. **a** The extent of hepatic inflammation was markedly attenuated with BTS: hepatocyte ballooning and Mallory–Denk bodies in the livers of GTG + HF fed mice were absent in BTS treatment livers. *Filled arrow* ballooning hepatocyte, *arrow* Mallory–Denk body. **b** Oxidative stress and TNF-α expression: BTS reduced hepatic oxidative stress as shown by reduction of numbers of nuclei stained positive for 8-OHdG in GTG + HF mice. BTS similarly reduced hepatic TNF-α mRNA expression. *Triple asterisk* $p < 0.001$, *asterisk* $p < 0.01$ vs GTG + HF, *Triple plus symbol* $p < 0.001$ vs 2 %BTS, $n = 6$

Table 1 Physiological and biochemical analyses in mice treated with BTS

	SC ($n = 6$)	GTG + HF ($n = 6$)	2 %BTS ($n = 6$)	5 %BTS ($n = 6$)
AST (U/L)	86 ± 11	310 ± 114	271 ± 27	170 ± 46*
ALT (U/L)	31 ± 9	514 ± 170	433 ± 37	299 ± 133*
TG (mg/dL)	36 ± 7	37 ± 13	52 ± 11*	49 ± 8

Serum ALT and AST levels were significantly reduced in mice treated with GTG + HF + 5 % BTS compared to the control GTG + HF group, *$p < 0.05$. There was no change in serum TG level between the GTG + HF + 5 % BTS and GTG + HF groups although TG was elevated by 2 %BTS compared to GTG + HF (*$p < 0.05$)

* $p < 0.05$ vs GTG + HF, $n = 6$

PPAR-γ effects may also function to increase serum adiponectin, since PPAR-γ agonists have been shown to positively regulate serum adiponectin independently of adipose tissue volume regulation [28, 29].

To now study the mechanisms of inhibition of liver lipid metabolism by BTS in the presence of the activated adiponectin signaling pathway, AdipoR1 signaling pathways and their target genes were examined. It is known that activated AdipoR1 signaling decreases expression of SREBP-1c [30], a key regulator of hepatic fatty acid synthesis [31, 32], through AMPK activation [33]. Here, we showed increased phosphorylation of AMPK by BTS in parallel with activated AdipoR1 signaling (Fig. 4c). Additionally, the phosphorylation of AMPK could also have been induced by activated PPAR-γ, in BTS treated mice, since as above BTS could also have PPAR-γ agonist like actions. Moreover, in the livers of mice treated with BTS, there was a reduced expression of SREBP-1c (Fig. 4b) in parallel with activated adiponectin signaling and phosphorylation of AMPK.

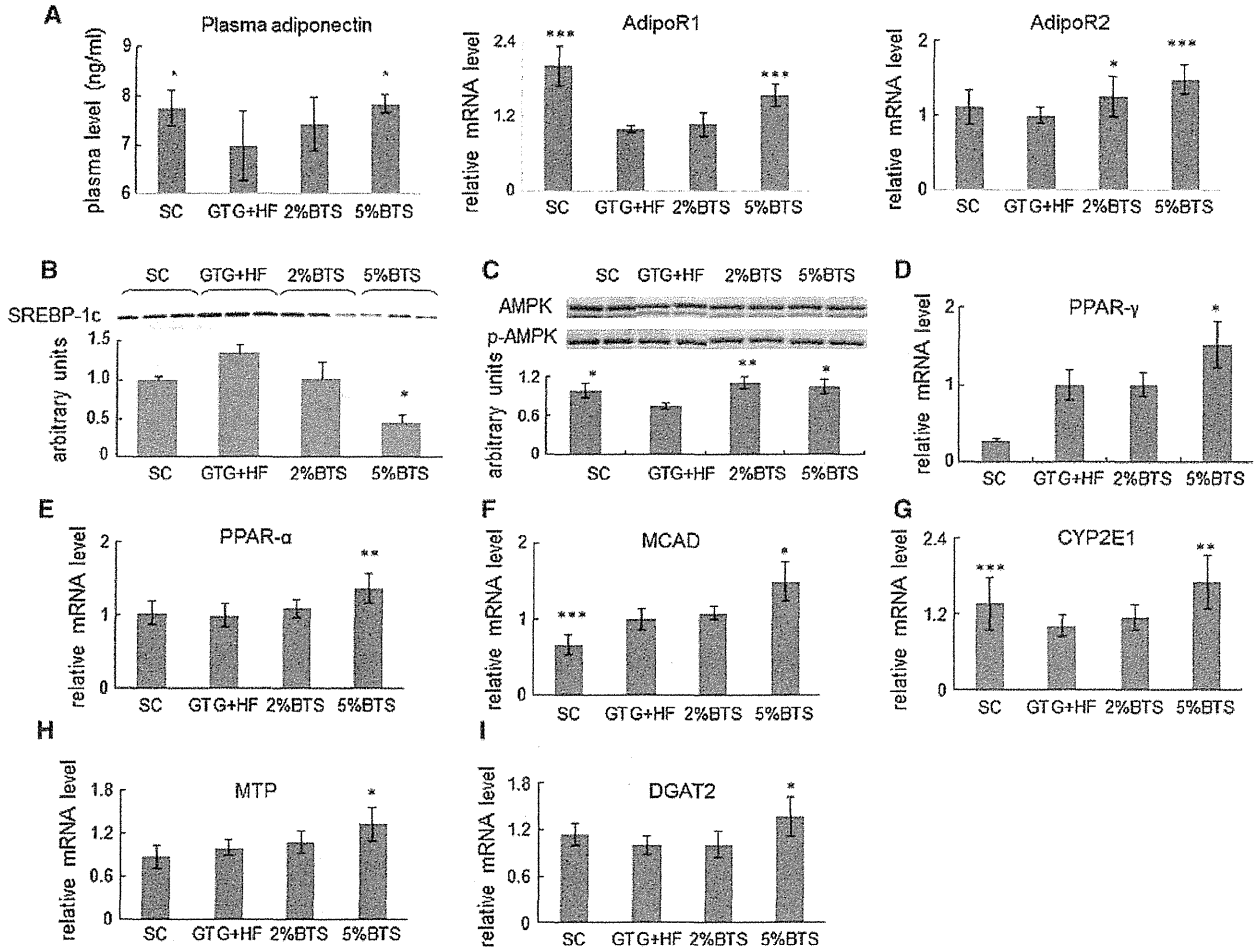

Fig. 4 BTS attenuates hepatosteatosis through activation of adiponectin. **a** Plasma adiponectin and expression of adiponectin receptors 1 (AdipoR1) and 2 (AdipoR2): plasma adiponectin levels and hepatic mRNA expression of AdipoR1 and AdipoR2 were dose dependently increased by BTS. *Asterisk* $p < 0.05$, *triple asterisk* $p < 0.001$ vs GTG + HF, $n = 6$. **b–d** AdioR1 signaling and target genes expression: BTS induced AdipoR1 signaling suppressed nuclear expression of SREBP-1c ($n = 4$) through phosphorylated AMPK ($n = 4$). Expression of PPAR-γ mRNA was also increased by BTS treatment ($n = 6$). *Asterisk* $p < 0.05$, *double asterisk* $p < 0.01$ vs GTG + HF. **e–g** Expression of PPAR-α and its target genes: Expression of PPAR-α mRNA was increase by BTS ($n = 6$). Expression of MCAD ($n = 6$) and CYP2E1 ($n = 6$), PPAR-α target genes, was similarly increased by BTS. *Asterisk* $p < 0.05$, *double asterisk* $p < 0.01$, *triple asterisk* $p < 0.001$ vs GTG + HF. **h, i** BTS promotes hepatic lipid export: MRNA expression of MTP ($n = 6$) and DGAT2 ($n = 6$) was increased by treatment with BTS. *Asterisk* $p < 0.05$ vs GTG + HF

TNF-α expression is also known to regulate expression of SREBP-1c through activation of AMPK [31, 34]. Therefore, the suppression of TNF-α expression by BTS, as observed here, could also have contributed to the decreased SREBP-1c expression. Taken together, therefore, the data here suggest that activation of AdipoR1 signaling and consequent suppression of SREBP-1c may be central mechanisms through which BTS attenuates hepatic steatosis.

BTS may also have enhanced hepatic fatty acid oxidation via AdipoR2 signaling, known to increase the expression of PPAR-α [24] and its fatty acid oxidation related target genes. Here, the expression of PPAR-α (Fig. 4e) and its target gene MCAD (Fig. 4f) and CYP2E1 (Fig. 4g), were increased by treatment with BTS. Therefore, activated fatty acids oxidation could also contribute to the attenuation of hepatic steatosis by BTS. Activation of fatty acid oxidation would be expected to increase production of reactive oxygen species (ROS) in the liver [35, 36]. BTS treatment here reduced ROS levels, probably contributing to the improved hepatic inflammation observed with BTS.

Additionally, MTP expression was confirmed here to be increased by treatment with BTS, possibly contributing to attenuation of steatosis by increasing hepatic export of TG (Fig. 4h). Interestingly, expression of DGAT2 was also increased by BTS treatment (Fig. 4i). Reduced hepatic accumulation of TG in BTS-treated livers may therefore have been due to increased MTP expression and increased expression of DGAT2 to reduce the content of FFA.

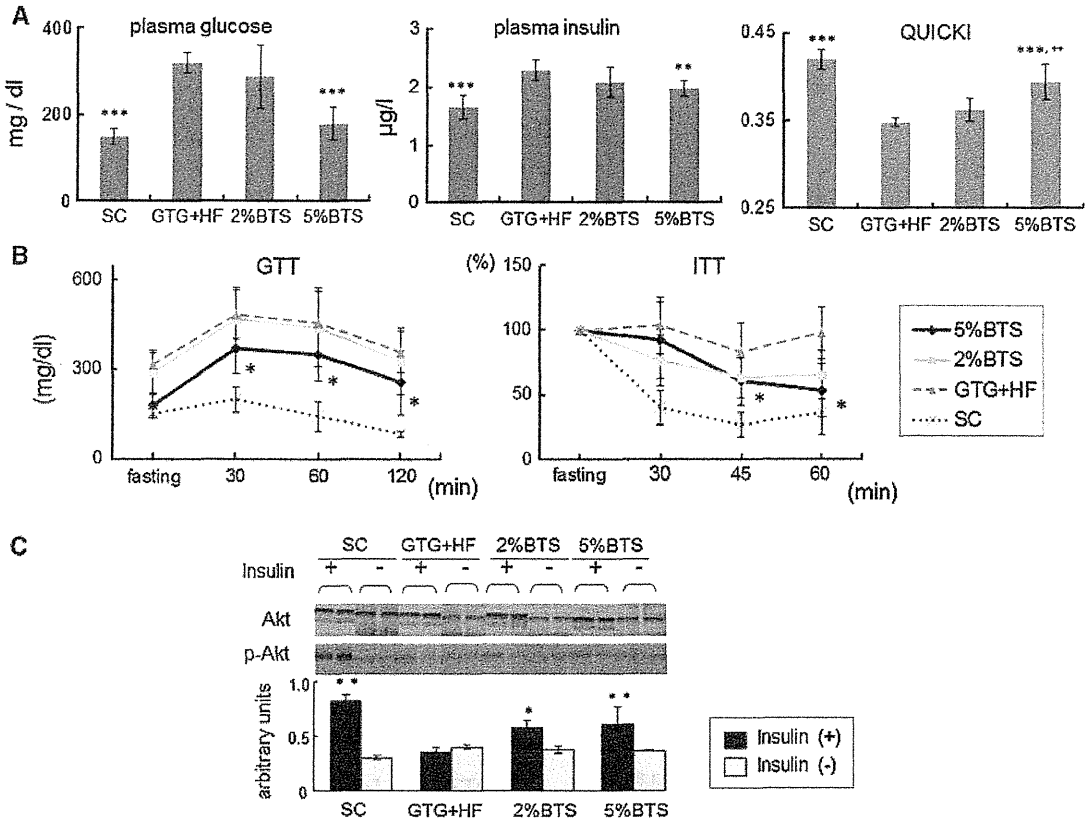

Fig. 5 Glucose intolerance and insulin resistance (IR) were attenuated by BTS. **a** Fasting plasma glucose, insulin levels and QUICKI: increased level of fasted plasma insulin and glucose were decreased, and QUICKI increased by treatment with 5 %BTS, and ITT confirmed severe IR *double asterisk* $p < 0.01$, *triple asterisk* $p < 0.001$ vs GTG + HF, *double plus symbol* $p < 0.01$ vs 2 %BTS, $n = 6$. **b** Glucose tolerance test (GTT) and insulin tolerance test (ITT): GTT revealed severe glucose intolerance was attenuated, and ITT confirmed IR was attenuated by 5 %BTS. *Asterisk* $p < 0.05$ vs GTG + HF, $n = 6$. **c** Involvement of Akt in BTS reduction of IR: phosphorylation of Akt was involved in the reduction of IR by BTS treatment as reflected by increased phosphorylation of Akt in the livers of BTS treated mice upon insulin administration. *Asterisk* $p < 0.05$, *double asterisk* $p < 0.01$ vs GTG + HF, $n = 4$

Furthermore, increased expression of DGAT2 might also have contributed to the reduction of ROS production by reducing hepatic FFA availability.

We next also investigated the effect of BTS on IR. GTG + HF induced elevation of fasting plasma glucose and plasma insulin were markedly reduced by BTS treatment, whilst QUICKI was markedly increased by BTS (Fig. 5a). In addition, ITT also was attenuated by BTS treatment. Furthermore, GTT showed that BTS remarkably attenuated severe glucose intolerance induced by GTG + HF (Fig. 5b). Phosphorylation of hepatic Akt after administration of insulin was reduced by treatment of BTS (Fig. 5c). The mechanisms through which BTS improves insulin sensitivity may involve Akt phosphorylation triggered through as yet uncertain mechanisms, but possibly involving induction of adiponectin signaling by BTS, since adiponectin is known to be involved in the suppression of hepatic gluconeogenesis and insulin secretion by activating AMPK [37].

In conclusion, therefore, BTS—a Japanese anti-obesity herbal (Kampo) medicine—is an effective preventive agent against the development of NASH. The challenge is now to identify the active components of BTS to allow its refinement and the rational design of more potent analogues.

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Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The association of insomnia with gastroesophageal reflux symptoms in biopsy-proven nonalcoholic fatty liver disease

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Abstract
Background It is suggested that nonalcoholic fatty liver disease (NAFLD), including nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH), can be associated with insomnia and gastro-esophageal reflux disease (GERD). The relationship between GERD and insomnia in subjects with biopsy-proven NAFLD was investigated.
Methods This study enrolled 123 patients with biopsy-proven NAFLD. Insomnia was assessed by the Athens Insomnia Scale (AIS), a self-assessment psychometric instrument designed to quantify sleep difficulty based on ICD-10 criteria; AIS scores ≥ 6 were considered positive for insomnia. GERD symptoms were evaluated using a frequency scale for the symptoms of GERD (FSSG); FSSG scores ≥ 8 were considered positive. Logistic regression models were used to evaluate the association of insomnia with GERD, after adjusting for potential confounders. Thirteen patients with GERD were treated with the proton pump inhibitor rabeprazole (RPZ; 10 mg/day), for 12 weeks.
Results Of the 123 patients, 76 (62 %) were female and 87 (71 %) were obese, with 34 (28 %) having AIS scores ≥ 6 and 31 (25 %) having FSSG scores ≥ 8. Liver biopsy revealed that 40 patients (33 %) had NAFL and 83 (67 %) had NASH. FSSG and AIS scores were similar in the two groups. HOMA-IR, FSSG scores and γGT (GGT) concentrations were significantly higher in insomniacs than in non-insomniacs. Logistic regression analysis demonstrated that FSSG score and GGT concentration were independently associated with insomnia. RPZ treatment resulted in significantly reductions in both AIS and FSSG scores.
Conclusions Nearly 30 % of patients with biopsy-proven NAFLD had insomnia, which was related to GGT and GERD and could be relieved by RPZ treatment.

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Abbreviations

BMI	Body mass index
GERD	Gastro-esophageal reflux disease
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis

Introduction

Nonalcoholic fatty liver disease (NAFLD) [1] is the most common chronic liver disease in many developed countries and results in serious public health problems worldwide. NAFLD includes a wide spectrum of liver diseases, ranging from nonalcoholic fatty liver (NAFL), which is usually benign, to nonalcoholic steatohepatitis (NASH), which may progress to liver cirrhosis (LC), hepatic failure and hepatocellular carcinoma (HCC) in the absence of significant alcohol consumption [2]. A large proportion of NAFLD patients are asymptomatic, but some occasionally experience fatigue, anxiety, and/or insomnia, resulting in a significant decrement in quality of life (QOL) [3]. In middle-aged Koreans, short sleep duration and poor sleep quality were found to be significantly associated with an increased risk of NAFLD [4]. Similarly, short sleep duration was associated with NAFLD in the general Japanese population [5]. However, the mechanisms underlying the association between insomnia and NAFLD remain unknown. Sleep is important to maintain body homeostasis, with sleep problems associated with all-cause mortality [6].

In addition to being associated with sleep problems, NAFLD was found, in two recent studies from Japan and Italy, to be associated with a high prevalence of the symptoms of gastro-esophageal reflux disease (GERD) [7, 8]. Evidence has emerged suggesting a link between metabolic syndrome, specifically obesity and visceral fat accumulation, and the onset of GERD. Studies throughout the world have shown that GERD is associated with sleep problems [9–14]. For example, a population-based study from Sweden showed positive associations among the presence of insomnia, sleeplessness, problems falling asleep, and risk of GERD [10]. In addition, an analysis of 19864 healthy adults in Japan found that poor sleep quality and irregular dietary habits were strong risk factors for high scores on the frequency scale for the symptoms of GERD (FSSG) [15]. Thus, it can be hypothesized that GERD symptoms may be responsible for insomnia in patients with NAFLD. To our knowledge, no study to date has assessed the prevalence of insomnia or GERD, or their association, in patients with biopsy-proven NAFLD.

Rabeprazole (RPZ), a proton pump inhibitor (PPI), is a potent and irreversible inhibitor of the H(+)/K(+)-ATPase gastric pump and is indicated for the treatment of GERD, Zollinger–Ellison syndrome, and duodenal and gastric ulcers. Moreover, the combination of RPZ and antibiotics is indicated for the eradication of *Helicobacter pylori*. RPZ is therefore expected to be effective in the treatment of GERD patients with sleep disturbances [16, 17]. This study was designed to evaluate the prevalence of insomnia and GERD in patients with biopsy-proven NAFLD; to compare the rates of insomnia and GERD in patients with NASH and NAFL; to determine independent predictors of insomnia, including FSSG score, among these patients; and to evaluate the effect of RPZ on insomnia.

Methods

Study population

The study included a total of 123 patients with well-characterized, liver biopsy–confirmed NAFLD who completed the FSSG questionnaire assessing symptoms of GERD and the Athens Insomnia Scale (AIS) questionnaire. All patients underwent biopsies at one of the seven hepatology centers included in the Japan Study Group of NAFLD (JSG-NAFLD): Center for Digestive and Liver Diseases, Nara City Hospital; Division of Gastroenterology, Yokohama City University Graduate School of Medicine; Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University; Department of Gastroenterology and Hepatology, Kochi Medical School; Department of Internal Medicine, Saga Medical School, Saga University; Department of Hepatology, Graduate School of Medicine, Osaka City University; and the Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine.

NAFLD was diagnosed based on liver biopsy findings of steatosis in ≥ 5 % of hepatocytes and the exclusion of other liver diseases, including viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, biliary obstruction, hemochromatosis, Wilson's disease, and α-1-antitrypsin-deficiency-associated liver disease. Patients consuming more than 20 g of alcohol per day, those with evidence of decompensated LC or HCC, those with psychiatric disorders or psychiatric drug users, and those taking PPIs and/or histamine H2-receptor antagonists were excluded. All patients provided written informed consent at the time of liver biopsy, and the study was conducted in conformance with the Declaration of Helsinki.

Laboratory and clinical parameters

Venous blood samples were taken in the morning after a 12-h overnight fast. Laboratory assays included blood cell counts and measurements of serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT), cholinesterase (ChE), total cholesterol, triglycerides, fasting plasma glucose (FPG), immunoreactive insulin (IRI), ferritin, hyaluronic acid, and type IV collagen 7S. These parameters were measured using the standard techniques of clinical chemistry laboratories.

Body mass index (BMI) was calculated as weight in kilograms/(height in meters)^2, with obesity defined as a BMI > 25 kg/m^2, according to the criteria of the Japan Society for the Study of Obesity [18]. Patients taking oral hypoglycemic medication, and those with a random glucose concentration > 200 mg/dl or a fasting glucose concentration > 126 mg/dl, were regarded as positive for hyperglycemia [19]. Patients with serum cholesterol concentrations > 220 mg/dl or triglyceride concentrations > 160 mg/dl were diagnosed with dyslipidemia. Patients taking antihypertensive agents and those having a resting recumbent blood pressure $\geq$ 140/90 mmHg on at least two occasions were regarded as having hypertension [20].

GERD score

The FSSG is a questionnaire widely used to diagnose GERD [21–24] and to evaluate the effectiveness of any treatment [21, 25].The FSSG consisted of 12 questions assessing the frequency of symptoms (never, 0; occasionally, 1; sometimes, 2; often, 3; and always, 4). Patients with FSSG scores ≥ 8 were considered positive for GERD; at this cut-off point, the FSSG had a sensitivity of 62 %, a specificity of 59 %, and an accuracy of 60 % in assessing GERD [21].

Insomnia scale

The intensity of sleep difficulty was evaluated using the AIS, a self-administered psychometric tool with high consistency, reliability and external validity (Table 1) [26, 27]. The AIS consists of eight items, five of which are used to assess insomnia, and the three used to assess well-being, functional capacity, and sleepiness during the day. The full eight-item version (AIS-8) was developed for clinical settings, while the five-item version (AIS-5) can be used to assess sleep quantity and quality. These first five questions (AIS-5) are used to assess difficulty with sleep induction, awakenings during the night, early morning awakening, total sleep time and overall quality of sleep. The last three items in the AIS-8 refer to

Table 1 Athens Insomnia Scale (AIS) [26]

Sleep induction (time it takes you to fall asleep after turning-off the lights)			
0: No problem	1: Slightly delayed	2: Markedly delayed	3: Very delayed or did not sleep at all
Awakening during the night			
0: No problem	1: Minor problem	2: Considerable problem	3: Serious problem or did not sleep at all
Final awakening earlier than desired			
0: Not earlier	1: A little earlier	2: Markedly earlier	3: Much earlier or did not sleep at all
Total sleep duration			
0: Sufficient	1: Slightly insufficient	2: Markedly insufficient	3: Very insufficient or did not sleep at all
Overall quality of sleep (no matter how long you slept)			
0: Satisfactory	1: Slightly unsatisfactory	2: Markedly unsatisfactory	3: Very unsatisfactory or did not sleep at all
Sense of well-being during the day			
0: Normal	1: Slightly decreased	2: Markedly decreased	3: Very decreased
Functioning (physical and mental) during the day			
0: Normal	1: Slightly decreased	2: Markedly decreased	3: Very decreased
Sleepiness during the day			
0: None	1: Mild	2: Considerable	3: Intense

Instructions this scale is intended to record own assessment of any sleep difficulty you might have experienced. Please, check (by circling the appropriate number) the items above to indicate your estimate of any difficulty, provided that it occurred at least three times per week during the last month

The period of the self-assessment may vary, depending on the design of a given study. Whenever the self-assessment pertains to a period other than that of the last month, the second sentence of the instructions should be rephrased accordingly