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Hybrid Training System Improves Insulin Resistance in Patients with Nonalcoholic Fatty Liver Disease: A Randomized Controlled Pilot Study

Sohei Iwanaga,[1,2] Ryuki Hashida,[1,2] Yoshio Takano,[3] Masafumi Bekki,[1,2] Dan Nakano,[4] Masayuki Omoto,[1,2] Takeshi Nago,[1,2] Takumi Kawaguchi,[4] Hiroo Matsuse,[1,2] Takuji Torimura[4] and Naoto Shiba[1,2]

[1]Division of Rehabilitation, Kurume University Hospital, Kurume, Fukuoka, Japan
[2]Department of Orthopedics, Kurume University School of Medicine, Kurume, Fukuoka, Japan
[3]Department of Physical Therapy School of Health Sciences at Fukuoka, International University Health and Welfare, Okawa, Fukuoka, Japan
[4]Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Fukuoka, Japan

Insulin resistance is associated with the progression of nonalcoholic fatty liver disease (NAFLD). Insulin resistance is regulated by various cytokines, including interleukin-6 (IL-6), a proinflammatory myokine, and selenoprotein P (SeP), a liver-derived secretory hepatokine. High levels of IL-6 and/or SeP have been shown to contribute to insulin resistance, and exercise is a first-line therapy for NAFLD. We have developed a hybrid training system (HTS): a neuromuscular electrical stimulation device to enhance exercise results. We aimed to investigate the effects of HTS on insulin resistance as well as serum IL-6 and SeP in patients with NAFLD. This is a randomized, single-blind (assessor), controlled trial. Subjects with NAFLD walked on a treadmill with or without HTS (9 subjects each) for 30 minutes three times a week for six weeks (HTS vs. control group; median age 45 vs. 45; male/female 5/4 vs. 6/3). We examined subjects before the first session and at the end of the final session. Serum SeP levels were measured by ELISA which measures the fragment of SeP. In the HTS group, HOMA-IR values were significantly reduced compared to the control group (Δ−0.71 vs. Δ0.05; $P < 0.05$). IL-6 and SeP levels in serum were also significantly reduced compared to that of the control group (IL-6; Δ−0.6 vs. Δ0.29 pg/mL; $P < 0.05$, SeP; Δ−1288.5 vs. Δ−435.4 ng/mL; $P < 0.05$, respectively). In conclusion, we propose that HTS improves insulin resistance by reducing serum IL-6 and SeP levels in patients with NAFLD.

Keywords: electrical stimulation; exercise; hepatokine; interleukin-6; myokine
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Introduction

An estimated 20 million Japanese have nonalcoholic fatty liver disease (NAFLD), which corresponds to 30% of the population (Eguchi et al. 2012). NAFLD is a chronic liver disease associated with obesity and metabolic syndrome. Insulin resistance is a feature of NAFLD and is associated with the progression of NAFLD-hepatocellular carcinoma, resulting in a poor prognosis (Romero-Gomez et al. 2017). Thus, lowering insulin resistance is an important therapeutic target for NAFLD. Physical inactivity is known as one of the causes of insulin resistance (Thyfault and Krogh-Madsen 2011), and exercise is the first-line therapy for patients with NAFLD (Hashida et al. 2017). There are two types of exercise: aerobic and resistance, both known to improve NAFLD (Hashida et al. 2017). However, exercise adherence in patients with NAFLD is poor because of exercise fatigue and discomfort (Hallsworth et al. 2011). Therefore, developing a method of more efficient exercise would be useful in the prevention of NAFLD.

The detailed mechanism of how exercise improves NAFLD remains unclear (Kawaguchi et al. 2011) because

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Correspondence: Ryuki Hashida, M.D., Ph.D., Department of Orthopedics, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan.
e-mail: hashida_ryuuki@med.kurume-u.ac.jp

exercise biology is complex and involves various metabolic and molecular changes that translate into changes in substrate utilization, enzyme activation, and ultimately, improvement in exercise performance (Huh 2018). Crosstalk between the liver, adipose tissue, and skeletal muscles might explain the effect of exercise on NAFLD. Each organ secretes a cytokine that mediates inflammatory or anti-inflammatory responses and metabolic adaptations. In adipose tissue, physical inactivity induces adipokine, a pro-inflammatory cytokine that leads to the development of insulin resistance (Iyer et al. 2010). Hepatocytes secrete liver-derived protein (hepatokines), and several are known to affect glucose and lipid metabolism. Selenoprotein P (SeP), one of the hepatokines, is involved in the development of insulin resistance and hyperglycemia (Misu et al. 2010). Muscle tissue secretes myokines, including interleukin-6 (IL-6), decorin, irisin and fibroblast growth factor 21 (FGF-21). The myokines improve energy balance and have the potential to be used as treatment for metabolic diseases, insulin resistance, and inflammation (Maalouf and El Khoury 2019). Thus, investigation of cytokines secreted through exercise might lead to the elucidation of a mechanism for improving NAFLD.

For strengthening muscles and improving function, neuromuscular electrical stimulation (NMES) is widely used (Balogun et al. 1993; Kagaya et al. 1995). NMES is also useful as an alternative to resistance exercise for people of advanced age or patients who are unable to perform high-intensity exercise (Banerjee et al. 2005). This leads to enhanced glucose metabolism and increased muscle hypertrophy (Hamada et al. 2003; Hasegawa et al. 2011). The combined application of electrical stimulation and exercise is said to be more effective than either electrical stimulation or exercise alone (Paillard 2008). We have developed a hybrid training system (HTS) of an NMES device that can be worn by users walking on a treadmill. Previous studies have suggested that combining voluntary exercise with NMES increases oxygen uptake by 9.1% compared to a group exercising without NMES, showing that exercise with NMES is more efficient (Bekki et al. 2019). Patients with NAFLD are disinclined to perform medium or high-impact exercise. Therefore, HTS would be useful to them because they would benefit more from lower impact activities, such as walking.

However, it remains unclear whether HTS helps improve hepatic steatosis and metabolism in patients with NAFLD. We aimed to investigate the synergetic effects of NMES and treadmill walking on insulin resistance as well as its effects on levels of adipokine, myokine, and hepatokine in patients with NAFLD.

Subjects and Methods

Subjects

This is a randomized, single-blind (assessor), controlled trial. This study was conducted to investigate the effects of using HTS with walking on a treadmill, in subjects with NAFLD. The Ethics Committee of Kurume University approved the clinical design of the study protocol (approval ID:17024). The study was registered in the University Hospital Medical Information Network Clinical Trial Registry (No.UMIN000028810).

The investigators informed subjects about exercise being first-line therapy for patients with NAFLD and explained the study procedures to the subjects who then gave their written informed consent to participate.

We followed the protocol for diagnosing NAFLD as described in the "Evidence-based clinical practice guidelines for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis" published by the Japanese Society of Gastroenterology in cooperation with the Japan Society of Hepatology (Watanabe et al. 2015). The research period was May 31, 2017 to February 28, 2019. We recruited subjects by poster from July-October 2017. The registration period was from July to December, 2017. Subjects did not have a habit of exercise, had been indicated as at risk for life-style related diseases by a specific medical checkup, and had not received any medical treatment. Forty-six participants applied. Following approval, informed consent was obtained from 43 people who had reviewed the goals of the study and agreed to participate. Exclusion criteria comprised evidence of other liver diseases such as chronic hepatitis C, chronic hepatitis B, autoimmune hepatitis, primary biliary cirrhosis, alcoholic liver disease, high alcohol consumption (> 20 g of alcohol/day), use of corticosteroids, tamoxifen, herbal medicines or anti-diabetic agents, heart or kidney disease and any other pre-existing medical conditions that might prevent participation in the exercise program. Participants were randomized with a 1:1 allocation using random block sizes of 4 using a 1:1 random generator. The HTS group or the control group were assigned to consecutive number according to the randomization. The allocation sequence was sequentially numbered, opaque, sealed and stapled envelopes, and it. Thus, 24 subjects with NAFLD were enrolled in this study and randomly placed into the HTS group (n = 12) or the control group (n = 12) (Fig. 1).

Training protocol

Subjects walked on the treadmill with (HTS group) or without (control group) HTS for 30 minutes three times a week for six weeks. We examined subjects before the first session and at the end of the final session. We selected the study protocol from a systematic review of exercise in NAFLD (Hashida et al. 2017). The subjects were instructed to continue in their daily activities as usual but were prohibited from starting any new physical activities such as resistance training or other activities that would improve their physical strength. During the walking exercise, an assistant was always present to provide guidance and monitoring so that the exercise was performed safely and correctly. Every exercise session began and ended with a 7-minute stretching session supervised by the assistant.

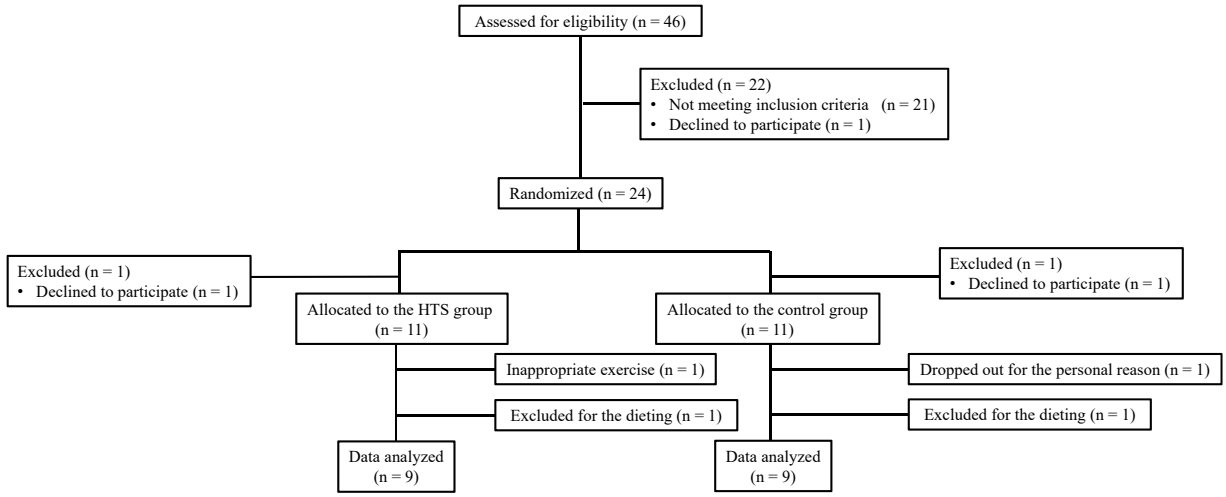

Fig. 1. Diagram of participant flow throughout the study.
46 applicants were reduced to 18 for various reasons and divided into two groups.
HTS, hybrid training system.

Hybrid training group

The electrical stimulator (EU-JLM50S, Panasonic Corporation, 1006 Ohaza-Kadoma, Kadoma City, Osaka, Japan) was a household medical device that included a wrapping cloth to fix the device on the legs and acceleration sensors or electrodes affixed to the skin that provided a constant voltage stimulus (regulated voltage) to both legs of the subjects as they walked on a treadmill.

Stopping criteria

Subjects could elect to discontinue at any time. If participants either appeared or reported to experience (a) physical deconditioning, (b) pain, or (c) inability to complete the protocol, their participation was discontinued.

Outcome measurements

Clinical data values to help assess hepatic steatosis, insulin resistance, and serum myokines were collected with details described below. The assessor was blinded to the randomized allocation of participants to minimize bias in collecting outcome measures.

Body weight, anthropometry, body composition

We assessed body weight, anthropometry, and body composition by using an eight-polar direct segmental multi-frequency-bioelectrical impedance analyzer (BIA; InBody720, Biospace, Tokyo, Japan), which is approved as a medical device in Japan, USA, Canada, Korea, and China. The reliability of InBody has been validated for Japanese people (Torimoto et al. 2009).

Hepatic Steatosis

Controlled attenuation parameter (CAP) has been used in the diagnosis of fatty liver. CAP based on FibroScan is a promising tool for noninvasive semiquantitative assessment of liver fat content (de Ledinghen et al. 2012). For the instantaneous assessment of liver steatosis, we used CAP to measure ultrasound attenuation at the mid-range frequency of the FibroScan M probe (3.5 MHz), with values ranging from 100 to 400 dB/m (de Ledinghen et al. 2014). CAP cut-off value for detecting significant hepatic steatosis was 232.5 (Masaki et al. 2013).

Laboratory determinations

Blood samples were taken in the morning after fasting from 9 pm the day before. Blood glucose, hemoglobin A1c, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using standard clinical methods (Department of Clinical Laboratory, Kurume University Hospital) as previously described (Kawaguchi et al. 2004, 2007, 2009). Insulin resistance and beta-cell function were calculated on the basis of fasting levels of plasma glucose and insulin by using the method of homeostasis model assessment of insulin resistance (HOMA-IR) (Matthews et al. 1985).

Measurement of cytokine

Serum levels of adiponectin were measured using a Human Adiponectin ELISA kit (DRP300; R&D; Systems, Inc., Minneapolis, MN, USA). Serum levels of decorin were measured using a Human Decorin ELISA Kit (ab99998; Abcam plc., Cambridge, UK). Serum levels of irisin were measured using an Irisin ELISA Kit (EK06716; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). Serum levels of FGF-21 were measured using a Human FGF-21 ELISA Kit (DF2100; R&D; Systems, Inc., Minneapolis, MN, USA). Serum levels of IL-6 were measured using the chemiluminescence enzyme immunoassay

method (LUMIPULSE F; Fujirebio Inc., Tokyo, Japan). Serum levels of SeP were measured using a Selenoprotein P ELISA Kit (CSB-EL021018HU; CUSABIO, Wuhan, China) which measures a fragment of SeP.

Statistical analysis

There was no preliminary research evaluating the effect of use or nonuse of HTS on insulin resistance in aerobic exercise for patients with NAFLD. Therefore, we did not set the sample size as an exploratory study.

All the statistical analyses were performed by JMP Version 12.0 statistical software (SAS Institute Inc., Cary, NC, USA), and values of $P < 0.05$ were considered to be statistically significant in all cases. Differences in baseline characteristics between the groups and changes from baseline between the two training groups were tested by the Wilcoxon rank-sum test or the Fisher's test as appropriate. The Wilcoxon rank sum test was used to check changes from the baseline in each group. All data are expressed as median. The effect of hybrid training was evaluated by changes in the variable (Δ = variable after training − variable before training).

Results

Baseline characteristics

Table 1 shows the characteristics of the HTS and control groups. The groups did not any significant differences in age, gender, body mass index (BMI), or waist circumference, in biochemical tests or hepatic steatosis, or in IL-6, decorin, adiponectin, or SeP levels in serum.

Adherence and adverse events of HTS

All subjects completed all of the walking exercise without problems such as severe pain or fatigue. One subject of each group declined to participate in this study. One subject from the control group dropped out for a personal reason. One subject from the HTS group was excluded due to inappropriate exercise. Furthermore, we excluded one subject in each group for dieting infractions. Finally, nine subjects of each group concluded the follow-up assessment.

Effects of hybrid training on body weight, anthropometry, and visceral fat

No statistically significant differences between groups were found for body weight (Fig. 2A), abdominal circumference (Fig. 2B), or visceral fat (Fig. 2C).

Table 1. Characteristics of subjects at baseline.

	Reference Value	Control group	HTS group	
		Median (IQR)	Median (IQR)	P
Number	N/A	9	9	
Age (years)	N/A	45.0 (40.0-51.5)	45.0 (34.0-50.5)	0.7223
Sex (female/male)	N/A	3/6	4/5	0.6287
Body weight (kg)	N/A	76.8 (64.7-77.9)	82.4 (62.2-87.2)	0.8598
Abdominal circumference (cm)	N/A	91.4 (89.9-97.2)	96 (88.1-101.15)	0.7911
SMI (cm^2/m^2)	N/A	8.02 (6.94-8.23)	7.66 (6.72-8.87)	0.7239
VF (cm^2)	N/A	128 (106-155.5)	144 (101-185.5)	0.5962
AST (U/L)	13-30	25 (19-29)	23 (21-31.5)	0.9647
ALT (U/L)	10-30	31 (24-47.5)	35 (28.5-48)	0.6270
HbA1c (%)	4.3-5.8	5.4 (5.3-5.6)	5.4 (5.25-5.5)	0.6182
Insulin (μIU/mL)	1.84-12.2	7.28 (5.74-14.05)	8.18 (7.46-14.45)	0.4268
Glucose (mg/dl)	70-109	99 (97.5-103.5)	96 (93.5-103)	0.2495
HOMA-IR		1.78 (1.43-3.48)	1.94 (1.66-3.59)	0.4799
FIB-4		0.64 (0.52-0.94)	0.75 (0.63-0.91)	0.3314
CAP (dB/m)		318 (256-351)	318 (260-356)	0.8250
IL-6 (pg/ml)		0.66 (0.295-1.395)	1.13 (0.73-2.145)	0.1221
Decorin (pg/ml)		9,294.8 (8,229.605-11,305.24)	8,826.19 (7,637-9,721.37)	0.3314
Adiponectin (ng/ml)		1,634.93 (1,135.955-3,020.02)	2,718.87 (1,634.11-4,435.28)	0.1577
Irisin (ng/ml)		6.15 (5.75-7.46)	6.24 (5.86-7.64)	0.9296
FGF-21 (pg/ml)		231.36 (174.78-384.78)	154.52 (133.69-468.25)	0.5365
Selenoprotein P (ng/ml)		3,426.59 (2,583.19-6,310.77)	4,996.74 (4,296.91-10,501.32)	0.0637

P values were calculated by the Wilcoxon rank sum test or the Fisher's test as appropriate.
IQR, interquartile range; N/A, not applicable; SMI, skeletal muscle index; VF, visceral fat; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HbA1c, hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; FIB-4, fibrosis-4 index; CAP, controlled attenuation parameter; FGF-21, fibroblast growth factor 21.

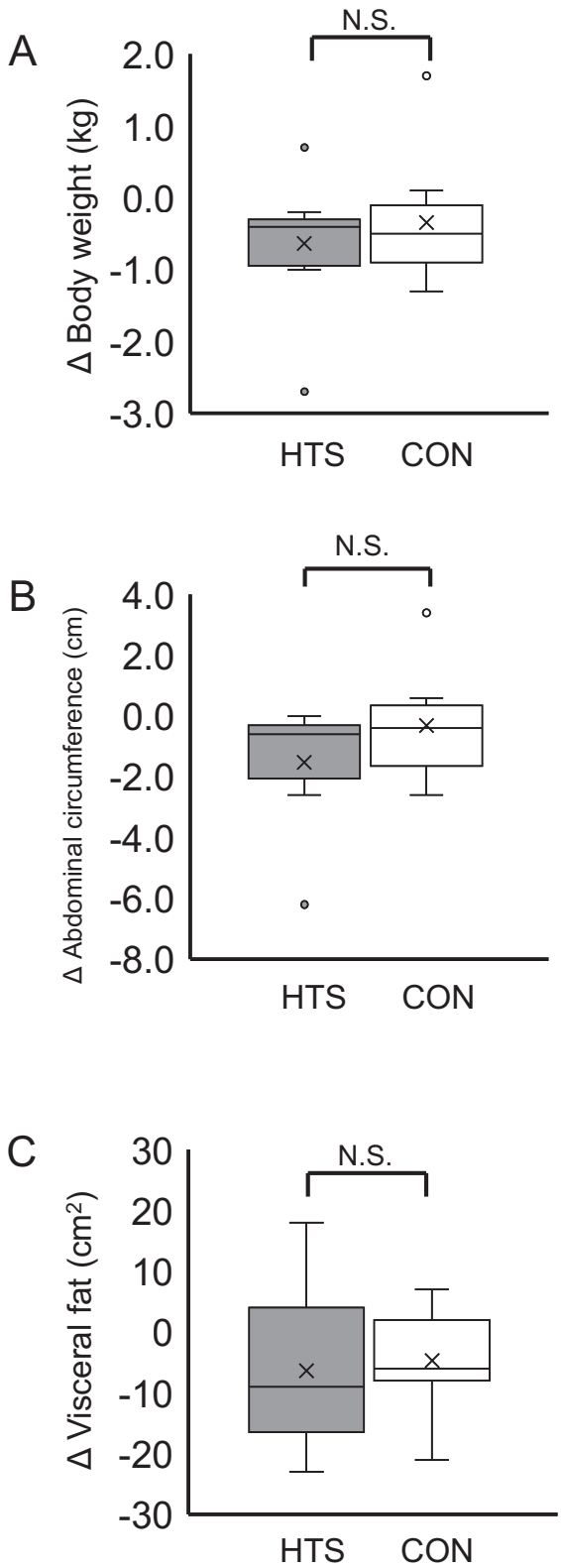

Fig. 2. Comparison of changes in body weight, anthropometry, and visceral fat before and after intervention.
The vertical axis shows the changes in body weight (A), abdominal circumference (B), and visceral fat (C). P values were calculated by the Wilcoxon rank sum test.
HTS, hybrid training system; CON, control.; N.S., not significant.

Effects of hybrid training on serum ALT levels and hepatic steatosis

Changes in AST levels in serum (Fig. 3A), ALT levels in serum (Fig. 3B), CAP values (Fig. 3C) and FIB-4 (Fig. 3D) were not statistically different between the groups.

Effects of hybrid training on insulin resistance

No statistically significant differences between groups were found for fasting blood glucose or hemoglobin A1c levels (Fig. 3E, F). However, compared to the control, the HTS group showed significantly lower insulin levels in serum (−2.98[−5.43-−0.03] vs. 0.01[−1.75-0.73] IU/mL, P = 0.0217) (Fig. 3G) and HOMA-IR values (−0.71 [−1.375-−0.09 vs. 0.05 [−0.71-0.17], P = 0.0273) (Fig. 3H).

Effects of hybrid training on levels of adiponectin, irisin, decorin, IL-6, and SeP in serum

In the analysis for cytokines, there was no significant difference in changes in serum adiponectin, decorin, irisin or FGF-21 levels between the control and the HTS groups (Fig. 4A-D). However, compared to the control group, levels were significantly reduced in serum for IL-6 (−0.6[−0.91-0.01] vs. 0.29[−0.16-0.71] pg/mL; P = 0.0341) (Fig. 4E). Moreover, SeP levels in serum were also significantly reduced compared to that of the control group (−1288.47[−1795.57-−816.34] vs. −435.43[−825.24-1.975] ng/mL; P = 0.0273) (Fig. 4F).

Discussion

Our study demonstrated that 30 minutes of walking on a treadmill with HTS three times a week for six weeks significantly lowered HOMA-IR values and reduced IL-6 and SeP levels in serum, compared to the control group. Thus, treadmill walking combined with HTS may synergistically lower insulin resistance by helping to inhibit myokine and hepatokine production in patients with NAFLD.

It is known that the combination of electrical stimulation and exercise improves insulin sensitivity. In a previous study, cycling ergometer exercise combined with electrical stimulation lowered HOMA-IR values and improved glucose metabolism (Chilibeck et al. 1999; Mohr et al. 2001). In our study, we were the first to demonstrate that electrical stimulation combined with treadmill walking reduces insulin resistance in patients with NAFLD.

There are various factors related to a reduction in HOMA-IR values, and we propose the following to explain the reduction in insulin resistance, which we believe is related to the electrical stimulation effect on muscles. Muscle fibers are classified as Type I (slow-twitch), Type IIa (fast-twitch A), and Type IIb (fast-twitch B) fibers (Kawaguchi et al. 2011). Type IIb fiber is only associated with anaerobic glycolysis. On the other hand, Type IIa fiber is associated with both anaerobic and aerobic glycolysis. Thus, Type IIa fiber is more sensitive to insulin than is Type IIb fiber (Kawaguchi et al. 2011). Patients with obesity and Type 2 diabetes mellitus show a higher proportion of Type

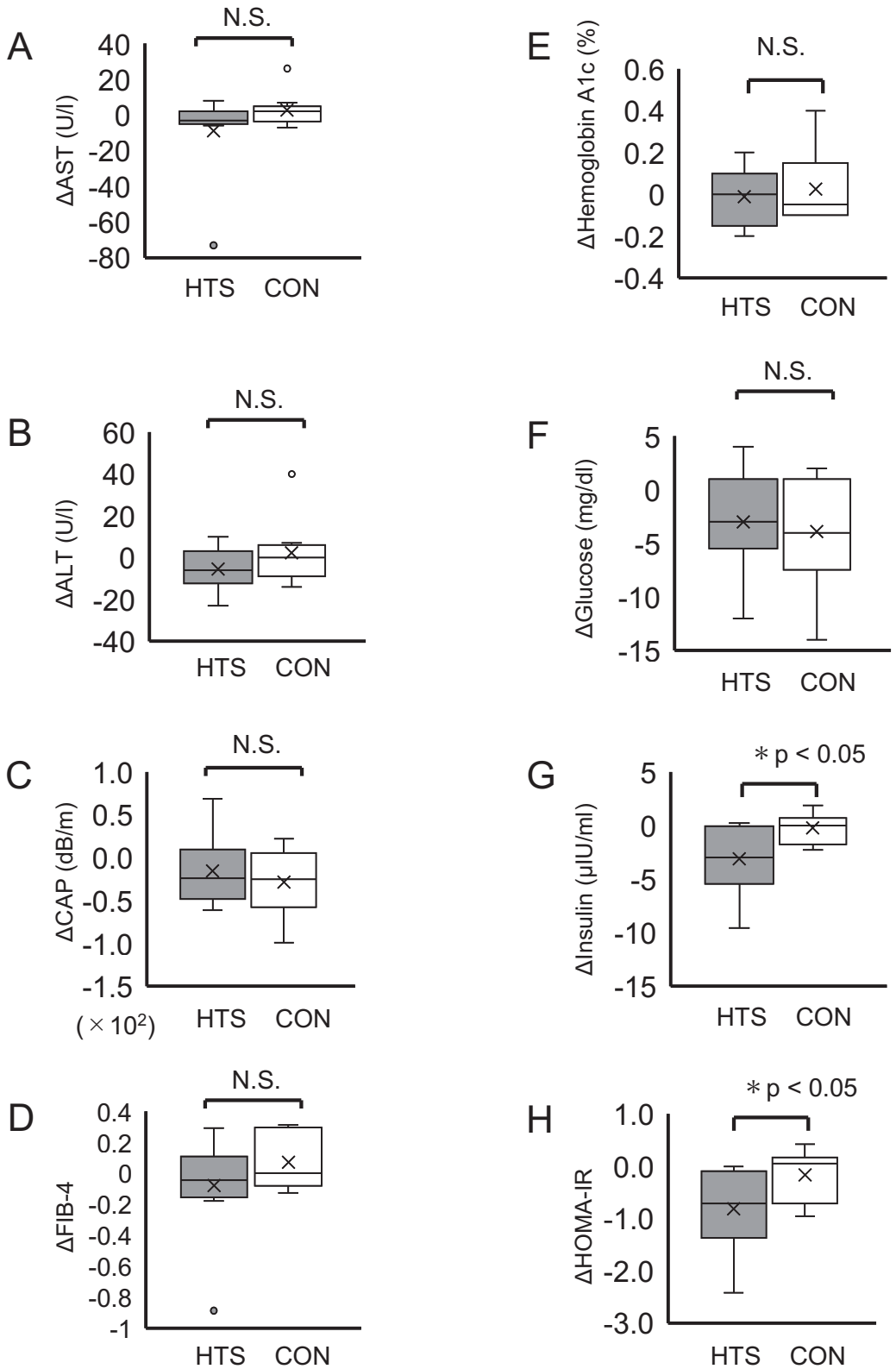

Fig. 3. Comparison of changes in laboratory determinations before and after intervention.
The vertical axis shows the changes in AST (A), ALT (B), CAP (C), FIB-4 (D), Hemoglobin A1c (E), Glucose (F), Insulin (G), and HOMA-IR (H). P values were calculated by the Wilcoxon rank sum test.
HTS, hybrid training system; CON, control; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CAP, controlled attenuation parameter; FIB4, fibrosis-4 index; HOMA-IR, homeostatic model assessment of insulin resistance; N.S., not significant.

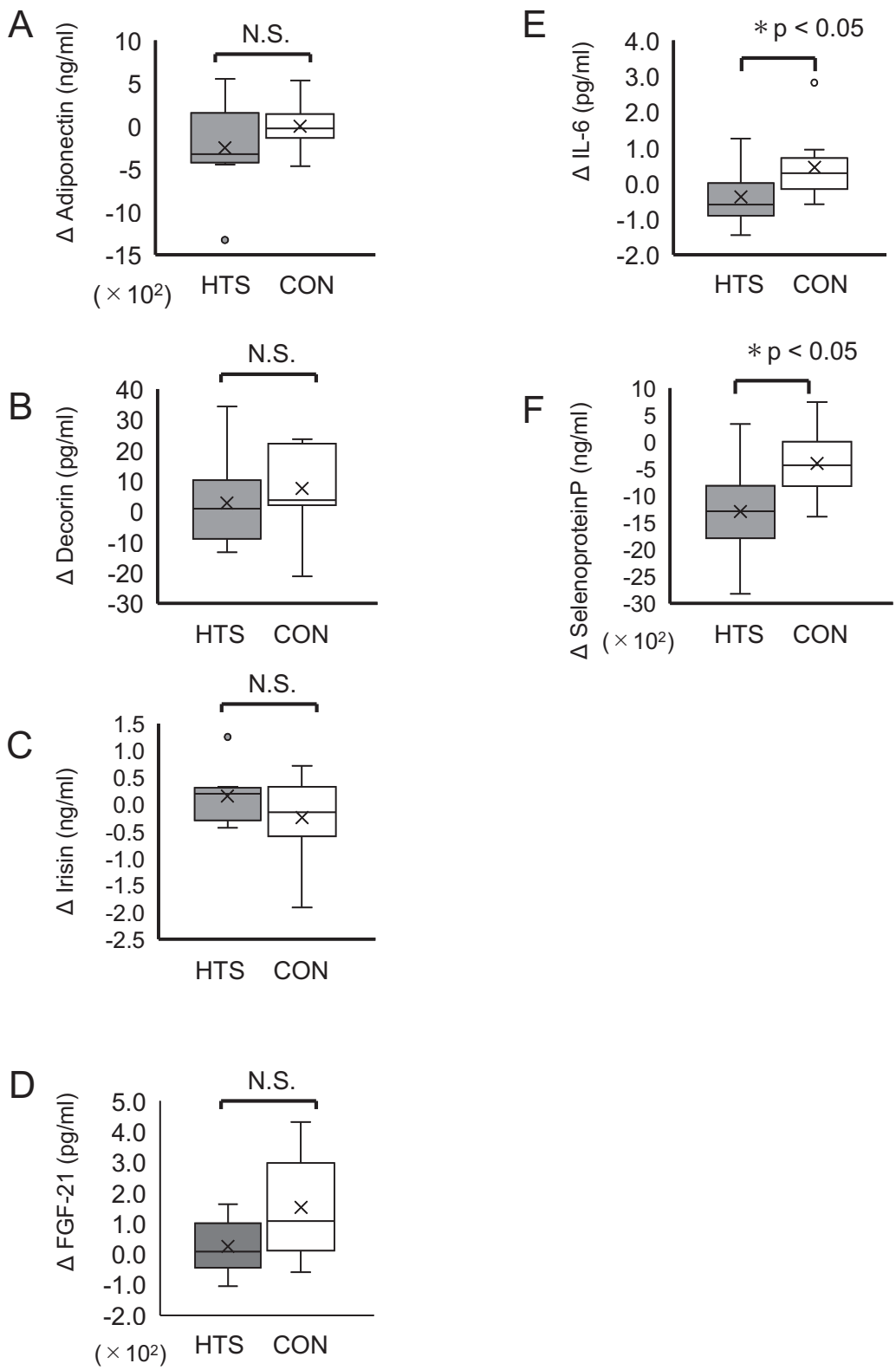

Fig. 4. Comparison of changes in levels of adipokine, myokine, hepatokine before and after intervention.
The vertical axis shows the changes in Adiponectin (A), Decorin (B), Irisin (C), FGF-21 (D), IL-6 (E), and Selenoprotein P (F). P values were calculated by the Wilcoxon rank sum test.
HTS, hybrid training system; CON, control; FGF-21, fibroblast growth factor 21; N.S., not significant.

IIb fibers compared to healthy people (Kawaguchi et al. 2011). Mohr et al. (1997)show that electrical stimulation of muscles transforms muscle fibers from Type IIb to Type IIa. Thus, they concluded that the electrical stimulation of muscle might reduce insulin resistance. Another group found that electrical stimulation significantly activated Type II fiber more than volitional exercise did (Yanagi et al. 2003).

Skeletal muscles provide inflammatory cytokines, known as "myokines". These locally generated myokines (e.g., IL-6) are linked to the inflammatory process. It is known that IL-6 is implicated in lowered energy metabolism and is also an inflammatory cytokine that is elevated in patients with NAFLD (Kawaguchi et al. 2011). Moreover, Zhang et al. (2018) showed that insulin resistance is associated with IL-6. In a clinical study that included patients with nonalcoholic steatohepatitis, aerobic exercise training three times a week for three months reduced IL-6 levels in serum and improved liver function (Abd El-Kader et al. 2014).

In our study, the HTS group had significantly lower IL-6 in serum than did the control group. On the other hand, we previously showed that HTS during knee extension exercise resulted in lower IL-6 levels in serum which was associated with a decrease in HOMA-IR (Kawaguchi et al. 2011). In the Kohut study, exercise resulted in reduced IL-6 levels in serum due to IL-6 mRNA suppression in skeletal muscle regardless of the exercise type (Kohut et al. 2006). Muscle contractions decrease the number of secretory vesicles containing IL-6 which in turn decreases levels of IL-6 in serum (Lauritzen et al. 2013). Electrical stimulation induces activation of both fast twitch muscle and slow twitch muscle movements, regardless of exercise intensity (Adams et al. 1993) and lowers IL-6 levels in skeletal muscle, resulting in the reduction of insulin resistance. Considering this information, we propose that the lower IL-6 in serum that we obtained in the HTS group might explain the lower HOMA-IR values, suggesting that HTS can be used to improve insulin resistance in patients with NAFLD.

In this study, we showed for the first time that the use of electrical stimulation with 6-weeks of treadmill walking decreased SeP in serum. SeP is a hepatokine that affects lipid and glucose metabolism and is reported to be elevated in people with Type 2 diabetes, prediabetes, and NAFLD (Oo et al. 2018). Moreover, pre-training SeP levels are inversely correlated with aerobic capacity as an exercise result. High SeP concentrations in serum predict the ineffectiveness of exercise training in humans (Misu et al. 2017). Takahashi et al. (2018) reviewed the relationship between various exercises and hepatokine. According to the report, there was no change in SeP secretion with exercise training in mice or humans (moderate exercise intensity). We demonstrated for the first time a decrease in serum SeP levels by exercise with HTS. We investigated a correlation between changes in serum SeP levels and changes in various variables, however, changes in serum SeP levels were not correlated with changes in levels of BMI, visceral fat, hepatic steatosis, glucose, and insulin (data not shown). Although the regulatory factor for SeP remains unclear in this study, high intensity exercise is known to decrease serum selenium levels (Maynar et al. 2018). Suppression of selenium levels is reported to down-regulate SeP mRNA and protein levels in hepatocytes (Hill et al. 1996). Thus, one would think that exercise-induced reduction of selenium may have caused a decrease in serum SeP levels in the HTS group, which had a higher intensity than treadmill alone (control group).

It remains unclear the reason why there was no significant change in serum levels of irisin, adiponectin, decorin, and FGF-21 after exercise. In fact, Amaro Andrade et al. (2018) reported that serum irisin level was increased after exercising for 150 minutes/week for 1 year, and Becic et al. (2018) reported that an exercise protocol of 12 weeks or longer significantly increased adiponectin. In our study, exercise was performed 3 time/week for 6 weeks. Thus, duration, intensity, an/or frequency of exercise may be insufficient for releasing these cytokines.

There are several potential limitations of this study. We investigated several cytokines in this study. Cytokines are secreted from multiple organs. We could not prove that myokine and hepatokine are derived from the specific organs in this study. Although we recruited 22 subjects, dropout rate was 18%. This higher rate of dropout may have influenced the results of this study. We were able to show a significant lowering of HOMA-IR but did not find any improvement in hepatic steatosis in patients with NAFLD. Moreover, we did not manage the diet of the subjects. Thus, we need to carry out a longer-term investigation in which we also include management of the daily diet of patients. Finally, in 2018, Saito et al. (2018)showed that a sandwich assay system to specifically detect a full-length SeP have been more to be accurate than the fragment of SeP used in the study. The results of the amount of change in serum SeP levels in this study may have been different if the full-length SeP assay kit had been used. Further study will be needed in the future.

In conclusion, this study showed that HTS significantly reduced insulin resistance and exhibited decreases in IL-6 and SeP levels in serum of patients with NAFLD. In other words, HTS might improve insulin resistance through regulation of myokine and hepatokine in patients with NAFLD, which in turn may help inhibit the progression of NAFLD.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest in this study.

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Pharmaceutical Co., Ltd.

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