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Effects of Hochuekkito on Lenvatinib-Induced Fatigue in Mice

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Lenvatinib has been approved for treating various cancers; however, it exerts numerous adverse effects. Hochuekkito, a Japanese Kampo medicine, can alleviate these adverse effects. Here, we aimed to evaluate the effect of Hochuekkito on lenvatinib-induced chronic fatigue in a murine model. BALB/C mice were fed a control diet or a diet supplemented with 1.5% Hochuekkito for six weeks. On days 15–42, the mice were intraperitoneally injected with dimethyl sulfoxide or lenvatinib. Accordingly, the mice were divided into control/dimethyl sulfoxide, control/lenvatinib, 1.5% Hochuekkito/dimethyl sulfoxide, and 1.5% Hochuekkito/lenvatinib groups. Body weight and food intake were recorded daily. Nesting tests were performed once a week, and the serum interleukin-6 (IL-6) concentration was measured. Liver drug-metabolizing enzyme, CYP3A4, breast cancer resistance protein (BCRP), and P-glycoprotein (P-gp) levels were determined. The serum lenvatinib concentration and CYP3A4, BCRP, and P-gp levels did not differ significantly between the control/lenvatinib and 1.5% Hochuekkito/lenvatinib groups. The usage rate of nesting material on day 42 was higher in the 1.5% Hochuekkito/lenvatinib group than in the control/lenvatinib group ($p < 0.05$). The serum IL-6 level was lower in the control/dimethyl sulfoxide and 1.5% Hochuekkito/lenvatinib groups than in the control/lenvatinib group ($p < 0.05$). Overall, Hochuekkito may alleviate lenvatinib-induced fatigue through IL-6 inhibition.

Key words adverse effect, fatigue, Hochuekkito, lenvatinib, TJ-41

INTRODUCTION

Lenvatinib (E7080) has been widely used for treating certain types of cancer, including unresectable hepatocellular carcinoma (HCC).[1)] As an active multiple receptor tyrosine kinase inhibitor, lenvatinib inhibits a wide variety of growth factor receptors, including vascular endothelial growth factor,[1–3)] fibroblast growth factor,[1–4)] stem cell factor, platelet-derived growth factor α, and rearranged during transfection receptors.[2–4)] However, lenvatinib does not block the signal transduction pathways involving Src, epidermal growth factor receptor, or cyclin-dependent kinase 4.[5)] The metabolic elimination of lenvatinib occurs through several processes that involve CYP3A and ATP-binding cassette transporters, including P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP).[6,7)] Lenvatinib exhibits anti-tumor effects against several types of solid tumors, including thyroid cancer, HCC, renal cell carcinoma, and lung cancer;[8)] however, it also exerts adverse effects in many patients, which include decreased appetite, hypertension, fatigue, proteinuria, and palmar-plantar erythrodysesthesia.[3,9)] To date, there is no effective treatment for lenvatinib-induced adverse events. As patients often discontinue therapy due to lenvatinib-induced fatigue, alleviating this adverse effect is an urgent clinical need for improving the treatment outcomes of patients with HCC.

The Japanese Kampo medicine Hochuekkito Extract (HET), also known as *Bu Zhong Yi Qi Tang* in Chinese and *Bojungikgi-Tang* in Korean, is an herbal mixture that has been used extensively in China, Japan, and Korea for decades.[10)] HET has been shown to improve digestive system function and relieve fatigue and decreased appetite.[11)] HET has been widely known as the Kampo medicine which can exert immune effects.[12–14)] Moreover, HET reportedly improved daily activity performance in mice with chronic fatigue syndrome.[15)] Although the detailed underlying mechanism remains unclear, HET treatment has been reported to decrease the tumor-induced level of serum interleukin-6 (IL-6) in mice.[16)]

Evidence suggests that increased levels of chronic proinflammatory cytokines, particularly IL-6, are related to elevated fatigue or depression levels.[17,18)] Several studies have shown that the mechanisms underlying inflammation can promote tumor formation, proliferation, and metastasis.[19–22)] Therefore, IL-6 may be involved in cancer progression by promoting tumor initiation, growth, and metastasis.[20–22)] Elevated serum IL-6 levels lead to overactivation of JAK/STAT3 signaling, which is associated with HCC progression and poor prognoses, further supporting the involvement of IL-6 in cancer pathogenesis.[23,24)]

To further explore the effects of HET on lenvatinib-induced chronic fatigue and whether the underlying mechanism involves its regulation of IL-6, in this study, we established the first murine model of lenvatinib-induced chronic fatigue.

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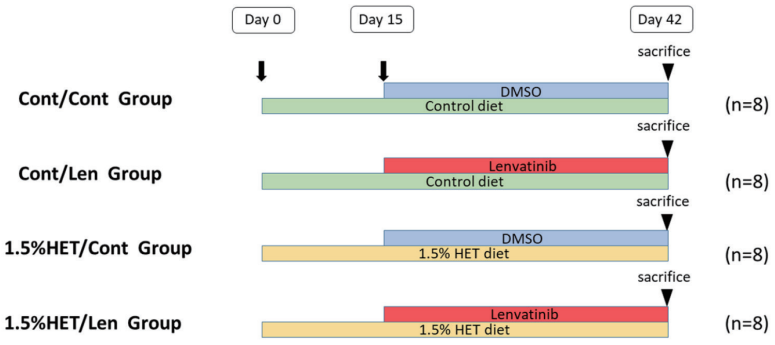

Fig. 1. Study Design

Cont: control; DMSO: dimethyl sulfoxide; HET: Hochuekkito Extract diet; Len: lenvatinib.

The mice were treated with lenvatinib with and without HET, and the influence on activity level, food intake, body weight, and the levels of drug-metabolizing enzymes and IL-6 were compared among the treatment groups. These findings may provide evidence to support the use of HET to alleviate the fatigue associated with lenvatinib treatment in cancer.

MATERIALS AND METHODS

Animals A total of 32 six-week-old, female, BALB/c mice (Japan SLC, Inc.) were housed in Hyogo Medical University Nishinomiya Pathological Model Research Center at room temperature (25 ± 1°C) under specific pathogen-free conditions. All animal experiments and related operations were performed according to internationally approved standards. The animal experiment protocol was approved by Hyogo Medical University (approval number 19-021).

Reagents Lenvatinib was purchased from ChemScene (Monmouth Junction, NJ, USA). Anti-P-gp (ab170904), anti-BCRP/ABCG2 (207732), anti-CYP3A4 (ab3572) antibodies (Abcam, Cambridge, UK), and β-actin antibody (A1978, Gene Tex Inc., CA, USA) were used for western blotting. Anti-CD31/PECAM-1 antibody was obtained from Relia Tech GmbH (Wolfenbüttel, Germany).

Murine Model of Lenvatinib-Induced Chronic Fatigue The mice were randomly divided into four treatment groups (n = 8 mice per group): the Cont/Cont group (n = 8) was fed a control diet and treated with dimethyl sulfoxide (DMSO), the Cont/Len group (n = 8) was fed a control diet and treated with lenvatinib, 1.5% HET/Cont group (n = 8) was fed a control diet supplemented with 1.5% HET and treated with DMSO, and 1.5% HET/Len group (n = 8) was fed a control diet supplemented with 1.5% HET and treated with lenvatinib. All mice were fed the corresponding diets for six weeks (days 1–42), including an initial two-week acclimation period with no treatment (days 1–14). After two weeks, treatment (DMSO or lenvatinib) was initiated and continued for four weeks (days 15–42). At the end of the experiments, all mice were euthanized. The body weight and food intake of the mice were recorded daily before lenvatinib administration. After four weeks of lenvatinib administration, the whole blood, middle lobe of the liver, small intestine, and colon were sampled for further analysis. The study design is schematically shown in Fig. 1.

HET Treatment A previous study suggests that HET should be administered in a diet with premedication in mouse experiments.[25] Therefore, the HET-containing diet was custom-manufactured by Oriental Yeast Co., Ltd. (Tokyo, Japan). It was prepared by supplementing the control diet with 1.5% (w/w) HET. HET, containing 10 different ingredients, was obtained from Tsumura & Co. (TJ-41, Lot No. 2200041020; Tokyo, Japan) (Table 1). The manufacturing method of HET complied with the Japanese Pharmacopoeia with respect to its components, composition, and quality; detailed information is available on the website of Tsumura & Co (https://www.tsumura.co.jp/english/kampo/07.html).

Lenvatinib Injection To obtain a final dose of 25 mg/kg, lenvatinib was first dissolved in DMSO (Sigma-Aldrich, MO, USA) with the addition of distilled water. Lenvatinib (25 mg/kg) was intraperitoneally administered to eight-week-old female BALB/c mice once a day for four weeks.

Measurement of Serum Lenvatinib and IL-6 Concentration After eight weeks of treatment, the whole blood of mice was drawn from the inferior vena cava under anesthesia into a 1.5-mL sterile tube and incubated before centrifugation. The supernatant was aspirated, and the serum was re-centrifuged under the same conditions. The serum samples obtained by centrifugation were stored at –80°C without repeated freezing and thawing to assess the lenvatinib concentration and IL-6 level. The serum lenvatinib concentration was determined using high-performance liquid chromatography, as previously described.[26] The serum IL-6 level was determined using an enzyme-linked immunoassay kit (Abcam, ab100712).

Western Blotting Liver specimens were homogenized with lysis buffer (T-PER; Pierce Biotechnology, IL, USA) supplemented with a protease/phosphatase inhibitor cocktail

Table 1. Composition of the HET Herbal Prescription

Ingredient	Proportion of prescription (%)
Astragali Radix	16.7
Atractylodes lancea rhizome	16.7
Ginseng Radix	16.7
Angelica Radix	12.5
Bupleuri Radix	8.3
Zizyphi Fructus	8.3
Aurantii nobilis pericarpium	8.3
Glycyrrhizae Radix	6.3
Cimicifugae Rhizoma	4.2
Zingiberis	2.0

(100X, #5872S, Cell Signaling Technology, MA, USA). Samples were divided into two groups: those that were boiled for 10 min and those that were not boiled. The proteins in the samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gradient gel; ATTO, Tokyo, Japan) and electrotransferred onto polyvinylidene difluoride membranes (MilliporeSigma, Burlington, MA, USA). Proteins of interest were detected by probing with primary antibodies, and targeted bands were visualized using a WSE-6100 LuminoGraphI system (ATTO). The specific antibodies for the boiled group were anti-BCRP/ABCG2 (ab207732, 1:1000), anti-CYP3A4 (ab3572, 1:2000), and anti-β-actin (A1978, 1:1000). Anti-P-gp antibody (ab170904, 1:1000) and anti-β-actin (A1978, 1:1000) were applied to the non-boiled group. The bands were visualized using anti-rat (#7074, Cell Signaling Technology) and anti-mouse (#7076, Cell Signaling Technology) secondary antibodies. ImageJ software (National Institute of Health, Bethesda, MD, USA) was used for Western blot densitometry band analysis.

Assessment of Mouse Activity To assess fatigue, a nesting test was performed once a week. Four pieces of nesting material were weighed and placed in cages. The residual nesting material was weighed after 24 h, and the usage rate was calculated by two different authors (JX and IN) in a blinded fashion.

Hematoxylin and Eosin (H&E) Staining Sections (4-μm-thick) were prepared from formalin-fixed organ samples (middle lobe of the liver, small intestine, and colon) harvested from the mice. The sections were placed on peeling prevention-coated glass slides (Matsunami Glass Ind., Osaka, Japan) and stained with H&E to determine any pathological changes.

Immunohistochemistry To examine liver sinusoidal endothelial cells (LSECs), the middle lobe of the liver specimens was fixed with immunohistochemistry zinc fixative (BD Biosciences, CA, USA) for at least 24 h. The paraffin-embedded samples were sectioned and pre-incubated with serum-free protein block (Agilent Technologies, CA, USA). The sections were then incubated with anti-CD31/PECAM-1 antibody (103-M104, Relia Tech GmbH) at a final concentration of 1.25 μg/mL. Histofine Simple Stain Mouse MAX-PO (Nichirei Biosciences Inc., Tokyo, Japan) was used for secondary antibody staining.

Statistical Analysis Data, presented as the mean ± standard error of the mean, were statistically analyzed using a free web software (www.gen-info.osaka-u.ac.jp/MEPHAS/tukey.html). Statistical comparisons between two experimental groups were performed using paired Student's *t*-tests. Tukey's post hoc test was used for pairwise comparisons among multiple groups. Results with $p < 0.05$ were considered statistically significant.

RESULTS

Preliminary Experiments to Optimize Lenvatinib Dosage As patients receive lenvatinib orally in the clinical setting, lenvatinib at a dose of 200 mg/kg was administered by oral gavage to the mice at the beginning of the experiment. However, despite increasing the lenvatinib dose, the blood concentration of lenvatinib was minimal. To improve the drug absorption efficiency and increase blood concentration of lenvatinib, the mice were administered lenvatinib intraperitoneally. To determine the appropriate lenvatinib dosage, a preliminary experiment was performed using five different concentrations of lenvatinib: 12.5, 25, 50, 100, and 200 mg/kg. After two weeks of lenvatinib treatment, some mice in the 200 mg/kg group had died. Additionally, pathological damage in the small intestine was observed in the 50, 100, and 200 mg/kg groups. After four weeks of lenvatinib treatment, mice in the 25 mg/kg group showed signs of fatigue without death or pathological damage in the small intestine. Mice in the 12.5 mg/kg group did not exhibit any adverse events. Therefore, to establish a stable murine model, we used the 25-mg/kg lenvatinib dosage regimen (Fig. S1).

Serum Lenvatinib Concentration and Drug-Metabolizing Enzyme Levels after 42 d of Treatment To investigate whether HET treatment affected the serum concentration of lenvatinib, blood was collected 24 h after the last lenvatinib administration. After 42 d of controlled diet and 28 d of lenvatinib injection, no significant differences in serum lenvatinib concentrations were observed between mice in the Cont/Len and 1.5% HET/Len groups ($p > 0.05$) (Fig. 2a).

In addition, we performed western blotting to examine the levels of different drug-metabolizing enzymes. Limited by the number of SDS-PAGE wells, we randomly selected six samples in each experimental group. No significant differences were observed in the protein levels of CYP3A4, BCRP, and P-gp between the Cont/Len and 1.5% HET/Len groups ($p > 0.05$) (Fig. 2b).

Effects of HET Treatment on Day 42 The body weight, food intake, and nesting behavior did not differ significantly between the Cont/Cont and 1.5% HET/Cont groups ($p > 0.05$). Compared with those in the Cont/Cont group, the mice in the Cont/Len group demonstrated decreased body weight and reduced use of nesting material ($p < 0.05$) (Figs. 3a and 3b). No significant differences in body weight, food intake, and nesting behavior were observed between the 1.5% HET/Len and 1.5% HET/Cont groups ($p > 0.05$). However, mice in the 1.5% HET/Len group showed higher use of nesting material than those in the Cont/Len group ($p < 0.05$) (Fig. 3b).

Tissue Examination after 42 d of Treatment H&E staining revealed no pathological changes in the livers of the mice in any group (Fig. S2). To further investigate whether long-term lenvatinib and HET administration affected or damaged the liver histology, liver samples were analyzed using immunohistochemistry with anti-CD31/PECAM-1 antibody to assess the LSEC quantity. No significant differences were observed among the treatment groups (Fig. S2).

As lenvatinib causes adverse events related to the digestive system (including decreased appetite and diarrhea), and intestinal diseases frequently have corresponding symptoms, we further examined the small intestine and colon samples using H&E staining after 42 d of treatment. No pathological changes were observed in the small intestine and colon samples of all groups (Fig. S2).

Serum IL-6 Levels The serum IL-6 level was higher in the Cont/Len group than in the Cont/Cont group ($p < 0.05$), whereas the serum IL-6 level was lower in the 1.5% HET/Len group than in the Cont/Len group. Nevertheless, no significant difference in the IL-6 level was observed between the Cont/Cont and 1.5% HET/Cont groups (Fig. 4).

DISCUSSION

Lenvatinib has been widely used to treat unresectable HCC and other types of cancer; however, many patients suffer from adverse events, including decreased appetite, hypertension,

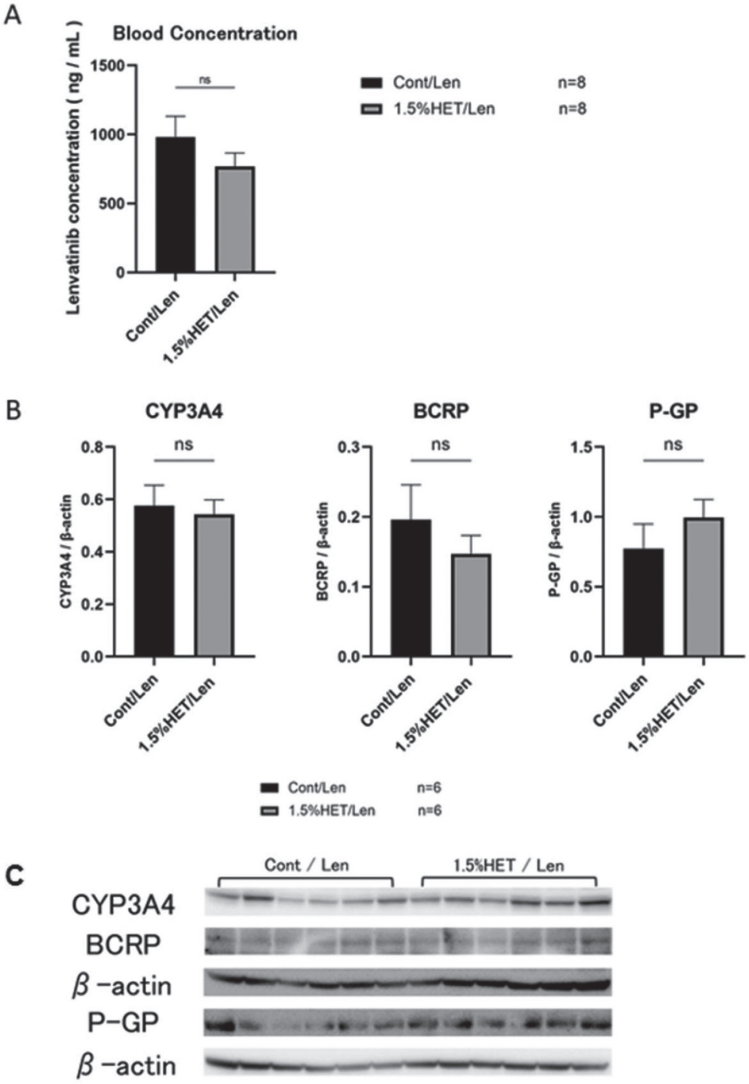

Fig. 2. Effect of HET on the Serum Lenvatinib Concentration and Drug-Metabolizing Enzyme Levels

Serum lenvatinib concentration (a) and lenvatinib-metabolizing enzyme (CYP3A4, P-gp, and BCRP) levels in the liver (b) of mice in the Cont/Len and 1.5% HET/Len groups at the end of the study (day 42). (c) Western blotting gel images of CYP3A4, P-gp, and BCRP in the liver of mice from each group are shown (ns = not significant). Limited by the number of SDS-PAGE wells, we randomly selected six samples from each experimental group.

fatigue, proteinuria, and palmar-plantar erythrodysesthesia, requiring dose reduction or treatment withdrawal. Evidence suggests that lenvatinib and sorafenib do not differ in terms of overall survival of patients with advanced HCC.[27] However, compared with sorafenib, lenvatinib improves the progression-free survival, objective response rate, and progression time of patients with unresectable HCC.[2] However, patients treated with sorafenib, an oral multi-kinase inhibitor frequently used to treat liver tumors, have a lower incidence of severe (grade ≥ 3) adverse events during treatment than those administered lenvatinib.[9] Moreover, the incidence of fatigue is lower in patients receiving sorafenib than in those receiving lenvatinib.[28] As the most frequently reported adverse event, fatigue is a concern for patients during lenvatinib therapy;[29,30] therefore, an effective treatment against the adverse events of lenvatinib is crucial. Here, we established a murine model of lenvatinib-induced chronic fatigue and examined whether HET could be used as an effective treatment against lenvatinib-induced adverse effects. We found that HET treatment did not affect the serum lenvatinib concentration. However, our experiment showed that HET treatment might reduce lenvatinib-induced chronic fatigue in mice. In addition, mice that received HET and lenvatinib had lower serum IL-6 levels than those that received only lenvatinib.

Lenvatinib is eliminated through enzymatic processes mediated by CYP3A4, aldehyde oxidase, P-gp, and BCRP,[6,7] as well as non-enzymatic processes.[31,32] Previous studies have demonstrated that administering HET may increase the quantity and activity of CYP3A4.[33] We found that administering HET did not significantly affect the liver CYP3A4, P-gp, or BCRP levels in mice. Furthermore, no significant difference was observed in the serum lenvatinib concentration between the Cont/Len and 1.5% HET/Len groups after two weeks of diet acclimation and an additional four weeks of 25 mg/kg of lenvatinib injection. Therefore, HET treatment did not decrease the serum lenvatinib concentration.

Nesting as a spontaneous, complex, highly motivated behavior can be observed throughout animal speices[34] and closely considered as activities of daily living (ADL) in human beings.[35] Nesting test as the noninvasive valuable, easy-to-

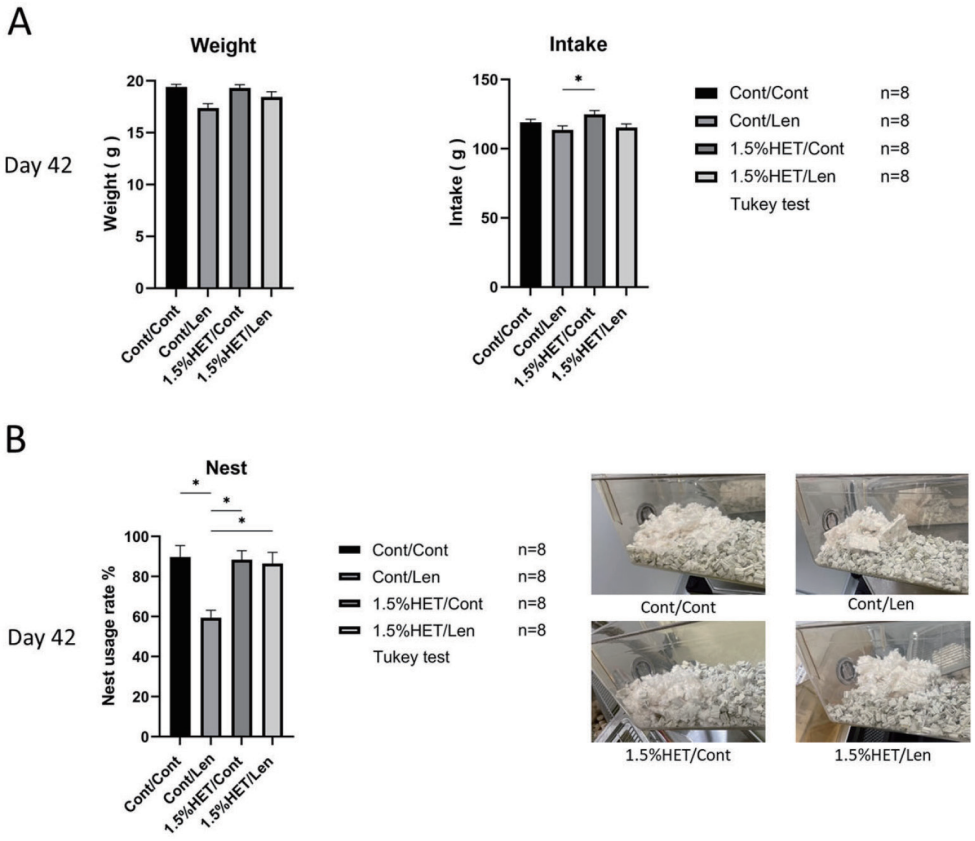

Fig. 3. Effect of HET Treatment on Body Weight, Food Intake, and Mouse Activity

(a) Body weight and food intake were analyzed in all groups on day 42 (following an additional four weeks of drug treatment). (b) Mouse activity in all groups was determined using the nesting test on day 42. A representative image after 24 h of nesting is shown for each group.

perform test is always used to evaluate the pain, distress, suffering, illness and ADL in mice.[35,36] Herein, considered the clearly relationship between ADL and fatigue, we performed nesting test to assess fatigue in our murine model. As higher use of nesting material mice was observed in the 1.5% HET/Len group than those in the Cont/Len group, it implies that the administration of HET might has the effect on lenvatinib-induced chronic fatigue.

There is a clear association between inflammatory responses and cancer development, including tumor promotion, initiation, and metastasis. Cytokines play a pivotal role in the association between inflammation and cancer, with potential to serve as therapeutic and preventive targets and prognostic factors.[37,38] Among the members of the interleukin cytokine family, IL-6 contributes to tumorigenesis and metastasis. IL-6 expression is highly upregulated in patients with chronic fatigue syndrome; its presence in the blood and cerebrospinal fluid is strongly associated with fatigue severity and progression.[39] Moreover, IL-6 administration has been shown to decrease nesting behavior in animal models.[40] As NF-κB is a key M1 macrophage transcription factor required for inducing genes encoding IL-6, activating NF-κB in macrophages is directly related to IL-6 production.[41] Several studies have illustrated that the active components of the HET formulation [42–51] contribute to the inhibition of NF-κB activation. Although the elevation of serum IL-6 has been observed in patients with HCC who had received lenvatinib treatment,[52] it has never been reported in animals without tumors during the lenvatinib trearment. In the present study, we confirmed that lenvatinib administration increases the serum IL-6 level, which was attenuated by HET treatment. After six weeks on a controlled diet followed by lenvatinib injection for four weeks, the use of nesting material was significantly higher in the 1.5% HET/Len group than in the Cont/Len group. Therefore, the effect of HET treatment on symptoms including fatigue may be associated with downregulation of IL-6 expression through NF-κB inhibition; however, further research is required to confirm this

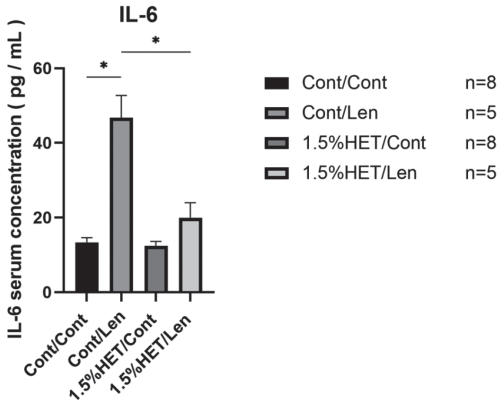

Fig. 4. Effect of HET Treatment on Serum IL-6 Levels

By the end of the study (day 42), the serum IL-6 level was determined in all groups using enzyme-linked immunosorbent assay. Limited by the volume of serum required for the assay, three samples each were excluded from the Con/Len and 1.5% HET/Len groups.

hypothesis. Moreover, given the association between IL-6 and cancer progression, HET treatment can potentially suppress tumor progression and metastasis by inhibiting IL-6 expression.

The present study has some limitations. Injecting lenvatinib into mice with HCC might cause fatigue due to cancer and lenvatinib administration, making it difficult to evaluate the effects of HET on lenvatinib-induced chronic fatigue. Therefore, to evaluate whether HET can alleviate lenvatinib-induced chronic fatigue efficiently, we did not perform these experiments on mice with HCC. Thus, further studies are required to determine whether administering HET specifically benefits lenvatinib-induced chronic fatigue in mice with HCC.

In conclusion, we established a stable murine model of lenvatinib-induced chronic fatigue and found that HET may reduce the chronic fatigue associated with lenvatinib treatment.

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Conflict of Interest Etsuro Hatano received support from Tsumura & Co. and payment for lectures, presentations, and speaker bureaus from Tsumura & Co. and Eisai. Naoki Fujitsuka and Sachiko Mogami are current employees of Tsumura & Co. The rest of the authors had no conflict of interest.

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