

1 Title

2 **Effects of ku-oketsu and seinetsu Kampo medicines on**
3 **rosacea-like skin symptoms induced by steroid in mice**

4 Natsu Watanabe¹, Wataru Matsunaga², Akinobu Gotoh^{3*}

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6 ¹ Cell and Gene therapy, Hyogo Medical University, Nishinomiya, Hyogo, Japan.

7 ² Joint-use Research Facilities, Hyogo Medical University, Nishinomiya, Hyogo, Japan.

8 ³ Department of Education for Medical Research Base, Hyogo Medical University,
9 Nishinomiya, Hyogo, Japan

10
11 * Correspondence: Akinobu Gotoh, M.D., Ph.D. gotoh@hyo-med.ac.jp.

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Abstract

Aim

Rosacea is a chronic inflammatory skin disease characterized by consistent erythema, papules, pustules, telangiectasias, and recurrent hot flashes, mainly on the cheeks, nose, and forehead. Many palliative treatment options are available for this condition; currently, no radical treatment option is available. However, improvement has been observed in some cases after the use of Kampo medicine. In this study, we aimed to analyze the effects of Jumi-Haidokuto (JHT) and a mixture of Keishi-Bukuryo-gan-ka-Yokuinin (KBY) and JHT on steroid-induced rosacea in mice.

Methods

Clobetasol propionate ointment was applied to the mice for 10 days, and then, the mice were provided food mixed with JHT and JHT + KBY for 28 days. Subsequently, the average velocity of capillary flow, area of the purpura, histopathologic features, and fecal metabolome were investigated.

Results

Recovery of the capillary flow velocity, reduction in the area of purpura, and restoration of the epidermal features were observed in JHT- and JHT + KBY-fed mice.

The improvement in circulatory disturbances observed after JHT and JHT + KBY

administration might have contributed to the reduction in telangiectasias and erythema.

Recovery of the skin barrier system by both Kampo medicine might be the key factor of ameliorating rosacea-like symptoms.

Key words: blood flow, Kampo, steroid, purpura, rosacea,

INTRODUCTION

Rosacea is an intractable skin disease whose symptoms are apparent mainly on the cheeks, nose, and forehead, which leads to impaired skin barrier function. It has various clinical manifestations, including persistent erythema, inflammatory papules, facial telangiectasia, pustules, and hot flashes. Moreover, skin dryness, scaling, extreme sensitivity, and burning sensations occur as the disease progresses [1].

The causal factors of rosacea are still unknown; however, an increase in Toll-like receptor 2 (TLR2) and antimicrobial peptide expression levels, both of which are involved in autoimmune disease development, is found at the lesion sites [2]. This increases the skin's sensitivity to external stimuli, and various factors such as ultraviolet light, exposure to Demodex, stress, lack of sleep, alcohol overconsumption, or consumption of spicy food may trigger inflammation, vasodilation, and hypervascularity [3].

In addition, long-term use of topical corticosteroids on the face results in steroid-induced rosacea or rosacea-like dermatitis [2]. The clinical manifestations of steroid-induced rosacea include thinning of the skin in addition to classical rosacea symptoms [4].

According to the results of a meta-analysis conducted using the findings of multiple trials, the global frequency of rosacea onset is 0.09–24.1% in adults, and a high frequency is observed among people of North European descent [5]. No extensive research has been conducted on the prevalence of rosacea in Japan; however, a survey of patients who visited dermatologists found that 0.22 % of the patients had rosacea and rosacea-like dermatitis [6]. Therefore, treatment of rosacea is an important global research issue, although it may not be a major problem in Japan.

To date, there are no established treatment options for rosacea. Options for rosacea treatment include oxymetazoline [7], brimonidine [8], azelaic acid [9], metronidazole [10], and ivermectin [11] as topical drugs, and doxycycline [12], isotretinoin [13], minocycline [14], β -blockers [15], and hydroxychloroquine [16] as oral drugs. However, the therapeutic effect of these drugs on rosacea is limited; thus, the American Academy of Dermatology describes these options as "management" options [17].

Some pharmaceutical-grade traditional Japanese medicines (Kampo) exhibit medicinal effects, including antioxidative and anti-inflammatory effects and blood flow improvement,

and have been reported to alleviate the symptoms of rosacea. "Oketsu" is a concept in traditional Japanese medicine that describes circulatory disturbances with vascular resistance and abnormalities in blood fluidity, and the symptoms of oketsu are similar to those of blood stasis. In Kampo medicine, pathological changes in the capillaries of patients with rosacea are considered "oketsu" and an inflammatory state [18]. The combined use of Kampo medicines that are anti-oketsu and antioxidative for the treatment of rosacea has been reported to improve rosacea symptoms [19].

Keishi-Bukuryo-gan-ka-Yokuinin (KBY) is a traditional Kampo pharmaceutical, generally prescribed for oketsu, which has been widely used for the treatment of menstrual irregularities. Administration of KBY, an anti-oketsu drug, effectively alleviated the symptoms of rosacea, possibly because it improved peripheral blood flow [19, 20]. Yokuinin (Coix seeds), a constituent of KBY, contains azelaic acid, which is used as a therapeutic agent for rosacea [21]. We investigated the therapeutic effects of KBY on rosacea-like symptoms and observed an improvement in skin capillary blood flow velocity, a reduction in the purpura area, and a recovery from skin thinning [22]. Although KBY showed a significant effect, its effect was insufficient to completely restore the symptoms. Therefore, we considered administering another Kampo medicine in combination with KBY to observe its effect on alleviation of rosacea-like symptoms.

Jumi-Haidokuto (JHT) is a pharmaceutical-grade traditional Kampo drug, which is popularly known as “seinetsu” (heat detoxification or heat clearing) drug. JHT is also prescribed for skin diseases such as urticaria, acne, and fungal infections. Therefore, we hypothesized that JHT alleviates rosacea symptoms through its anti-inflammatory and antioxidant effects [23].

In this study, we observed changes in blood flow and purpura in the skin following long-term oral administration of JHT alone and JHT + KBY in a mouse model showing rosacea-like symptoms. Moreover, histopathological analysis of pre- and post-JHT administered mice and fecal metabolome analyses were performed to analyze the pharmacological conditions. Overall, we aimed to investigate the efficacy of the combined use of JHT and KBY for the treatment of rosacea.

MATERIALS AND METHODS

Animals

Male hairless mice (Hos: HR-1; 4-week-old) were purchased from Hoshino Laboratory Animals, Inc. (Bandou, Ibaraki, Japan). They were housed under pathogen-free conditions at a temperature of 25 ± 2 °C, relative humidity of 55 ± 20 %, and a 12 h light: 12 h dark cycle, with lights on from 07:00 to 19:00 h daily. The mice had ad libitum access to food and water.

All protocols in this study were approved by the Animal Care and Use Committee of Hyogo Medical University (permit number: 21-004) and complied with the Animal Experiment Regulations of Hyogo Medical University. The experimental mice were euthanized by decapitation under deep anesthesia with isoflurane (Mylan EPD, Tokyo, Japan) according to the guidelines of the Japanese Society for Laboratory Animal Resources.

Experimental diet

In this study, the mice were fed a standard moderate-fat diet (MF diet; Oriental Yeast Co., Tokyo, Japan) containing powdered extracts of JHT (Kracie Pharma, Ltd., Tokyo, Japan) and KBY (Tsumura & Co., Tokyo, Japan). Both Kampo powders were prepared in compliance with good manufacturing practice guidelines.

The JHT (lot no. 2006261) used in this study was prepared as a spray-dried powder of hot water extracts from 10 crude drugs. Table 1 lists the crude herbs that constitute JHT. The mixture was extracted using hot water, dried using the spray drying method, and subsequently, the JHT extract was added to the MF diet at a concentration of 3.0 %.

KBY (lot no. 2190125010 and 2210125010) was prepared as a dried powder of hot water extracts from six crude drugs which are listed in Table 2

The mixture was then extracted with hot water and dried using the spray drying method.

The KBY extract was added to the MF diet at a concentration of 2.5 %.

JHT+KBY diet was prepared by pulverizing equal amounts of MF diet containing each kampo powder, mixing in a mixer, kneading with sterile distilled water containing 1% agar (Ina Food Industry Co. Ltd., Ina, Japan), and pelletizing.

Experimental design

The experimental timeline of this study is presented in Figure 1. After pre-breeding the mice for seven days, 2 g of steroid ointment (0.05 % clobetasol propionate ester; Sato Pharmacological Co., Tokyo, Japan) was applied to the dorsal side of the skin of each mouse daily for 10 days (days 0–9). The mice were fed JHT alone- and JHT + KBY-containing diet for 28 days (days 9–37), and the blood flow velocity and purpura were observed on days 0–37. Mice named as “no-Kampo” were treated with steroid ointment for 10 days but were fed standard MF diet. The control group was fed the standard MF diet without steroid treatment.

Video images of the mouse skin were recorded using a capillary flow scope (TOKU Capillaro; Toku Co., Tokyo, Japan), and video analysis was performed using the Capimetrics capillary analysis software (Toku Co.). The area of the purpura was measured using the ImageJ software. Images of the mouse skin were converted to grayscale (16-bit) and processed using the Gaussian Blur filter. Purpuras were detected in the images by automatically selecting

the threshold for differentiation between black and white regions, and the area of the black portion was measured automatically [23]. All experimental mice were euthanized on day 37.

Histopathological evaluation of dorsal skin

On days 9 or 37 of the experiment, dorsal skin samples of mice were excised under isoflurane (2-3%) anesthesia, and the skin samples were immediately fixed in 4 % formaldehyde at 4 °C for 48 h. To avoid prolonged suffering, mice whose skin was excised on day 9 were immediately euthanized and skin samples were not collected twice from the same mouse.

Analysis of fecal metabolome

Fecal metabolites were analyzed at Human Metabolome Technologies, Inc. (Yamagata, Japan). Capillary electrophoresis–mass spectrometry and liquid chromatography–mass spectrometry were performed to detect the metabolites; the analysis was mainly based on the metabolites mentioned in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database.

Statistical analysis

All values are expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed using t-test and analysis of variance (ANOVA), followed by Tukey's post-hoc test conducted using Free JSTAT version 22.0E. The results were considered statistically significant at $p < 0.05$.

RESULTS

Food consumption and body weight gain

Daily food consumption of experimental mice was 6.36 ± 0.66 g/day in the control group (standard MF diet), 6.35 ± 0.38 g/day in the JHT group and 6.65 ± 0.98 g/day in the JHT+KBY group; the values for the JHT+KBY group exclude the weight of water and agar used for forming pellets, therefore, actual consumption is twice this value. One-way analysis of variance did not detect significant differences in food consumption. The average body weights of the mice on days 9 and 37 were 24.58 ± 0.35 and 31.73 ± 0.31 g, respectively. Results of one-way ANOVA revealed no significant differences between the mean body weights of mice that underwent different experimental treatments. The average weights gained from day 9 to 37 were 6.89 ± 0.51 , 6.68 ± 0.19 , 7.75 ± 0.78 , and 7.20 ± 0.47 g in the control, no-Kampo, JHT, and JHT + KBY diet groups, respectively. Results of one-way

ANOVA revealed no significant differences between the average weights gained by the mice that underwent different experimental treatments. Taken together, the intake of kampo during the experimental period (28 days) was 5.00 (JHT+KBY) or 5.46 (JHT) g. The average body weight of the mice was 28 g and the kampo intake per kg of body weight was approximately 187.00 g/kg, which is approximately 40 times higher than the amount clinically administered to humans during 28 days (10 g/day for an adult weighing 60 kg).

Blood flow velocity in skin capillaries

The chronological changes in the average blood flow velocities of the skin capillaries are shown in Figure 2. Results of two-way ANOVA revealed a significant correlation between the average blood flow velocities observed in different experimental groups and the number of days ($F [3, 30] = 1.610$; $p = 0.0254$). Results of one-way ANOVA followed by Tukey's post-hoc test revealed significant differences between the blood flow velocities observed in different experimental groups ($F [3, 10] = 10.24$; $p < 0.001$). The average blood flow velocity in the no-Kampo group was significantly lower than that in the control group. In addition, the average blood flow velocities in the JHT + KBY and JHT groups were significantly higher than those in the no-Kampo group. On the other hand, no significant difference was observed between the blood flow velocity of the JHT+KBY group and the JHT group. Results of one-

way ANOVA revealed that on each day from day 21 to 30, the JHT + KBY group showed a significantly higher average blood flow velocity than that in the no-Kampo group. Additionally, on days 35 and 37, the average blood flow velocity in the JHT + KBY group was higher than that in the no-Kampo group.

Area of the skin purpura

Chronological changes in the average area of skin purpura within the observation field are shown in Figure 3A. Results of two-way ANOVA revealed a significant correlation between the areas of the skin purpura in different experimental groups and the number of days ($F(3, 30) = 2.353$; $p < 0.001$). Results of one-way ANOVA followed by Tukey's post-hoc test revealed significant differences between the areas of the skin purpura in different experimental groups ($F(3, 10) = 22.672$; $p < 0.001$). The average purpura area in the no-Kampo group was significantly larger than that in the control group. Moreover, the average skin purpura area was significantly higher in the JHT group than that in the control group. In contrast, the skin purpura areas in the JHT + KBY and JHT groups were significantly smaller than that in the no-Kampo group. In addition, the average area of skin purpura in the JHT + KBY group was significantly smaller than that in the JHT group. On individual measurement days, results of one-way ANOVA revealed that the purpuras were significantly larger in the

steroid-treated groups than that in the control group on day 9. After day 9, the JHT and no-Kampo groups generally showed larger purpura areas than those of the control group. In contrast, from day 16, significantly smaller purpura were observed in the JHT + KBY group than those in the no-Kampo group. Moreover, the average purpura areas of the JHT + KBY group were significantly smaller than those in the JHT group after day 14 and were comparable to the average purpura areas of the control group. Figure 3B shows a typical skin purpura. On day 9, prominent dark purpura was observed on the dorsal skin where clobetasol propionate had been applied, and similar dark purpura was observed on day 37 in the no-kampo group (white arrows). On the dorsal skin of the JHT group on day 37, the area of dark purpura was smaller than that on day 9 (or day 37 of the no-kampo group). The observed image of the JHT+KBY group on day 37 was very similar to that on day 0 (Fig.3B).

Histopathology

Typical histological images of dorsal skin samples of experimental mice are shown in Figure 4. On day 9, the mice showed peeling of the horny layer and thinning of the granular layer (A, arrows) following corticosteroid ointment application. On day 37, the dorsal skin of no-Kampo mice showed horny layer peeling, thinning of the granular layer, and insufficient recovery (B, arrows). However, JHT + KBY-fed mice showed an almost normal epidermis

and granular layer (C, arrow) on day 37, and the histological images of their skin samples were similar to those of control mice on day 37 (E). In contrast, the skin layers of the JHT group (D, arrows) were thinner than those of the KBY + JHT group on day 37 (C, arrows).

Results of metabolomic analysis

A comparison of the metabolomic analysis of feces from the KBY + JHT and no-Kampo groups is presented in Table 3. Metabolomic analysis revealed a significantly higher spermine content in the JHT + KBY group than that in the no-Kampo group ($p = 0.013$). Moreover, the malic acid ($p = 0.026$) and uracil ($p = 0.041$) content was significantly reduced in the JHT + KBY group compared to that in the no-Kampo group. Additionally, statistically significant changes ($p < 0.10$) in hydroxyproline, asparagine (Asn), and S-adenosylmethionine levels were detected. The levels of hydroxyproline and asparagine in the JHT + KBY group were significantly higher than those in the no-Kampo group, and S-adenosylmethionine levels in the JHT + KBY group were lower than those in the no-Kampo group.

DISCUSSION

In a previous study, we investigated the efficacy of KBY against skin damage in a mouse model of steroid-induced rosacea [22]. Administration of KBY for 37 days significantly

improved skin blood flow and reduced skin purpura and thinning in the model mice. However, the purpura area in the KBY-treated group was not reduced to a level equal to that of the control group, indicating that the therapeutic effect of KBY on purpura were insufficient [22]. Therefore, we investigated the efficacy of JHT, a typical anti-inflammatory drug, and JHY + KBY, a traditional Japanese medicine, in a glucocorticoid-induced rosacea-like mouse model by assessing capillary circulatory flow, purpura area, histopathological features of the dorsal skin, and fecal metabolome.

Blood flow velocity in the skin capillaries was significantly improved after treatment with both JHT alone and JHT + KBY. The purpura area of the skin was also reduced in the JHT alone and JHT + KBY groups. However, the purpura area in the JHT group significantly differed from that in the control group, and it can be concluded that JHT alone did not sufficiently restore the purpura area to a level equal to that of the control. Our previous results showed that KBY alone did not reduce skin purpura area to a level equal to that of the control [22]. In contrast, the purpura area in the JHT + KBY group was not significantly different from that in the control group. This fact suggests that a ku-oketsu agent (KBY) or seinetsu agent (JHT) alone is not sufficient for complete improvement of skin purpura and that synergistic effects of ku-oketsu agent and seinetsu agents are required.

The histopathological observations also revealed a marked recovery following skin damage

caused by the clobetasol propionate ointment in the JHT + KBY group. However, on day 37, the recovery efficacy in the JHT alone group was not similar to that in the JHT + KBY group. Moreover, our previous histopathological observations have shown that KBY alone ameliorates skin depilation [22].

The effects of Kampo medicines on the symptoms of rosacea in model mice are summarized in Table 4.

The recovery levels of blood flow velocities in the skin capillaries after the application of clobetasol propionate were almost similar in the KBY, JHT, and JHT + KBY groups. The highest recovery of skin purpura area was observed in the JHT + KBY group. Moreover, the histopathological symptoms were most effectively reversed in the JHT + KBY group. Our present and previous studies have shown that JHT, KBY, or JHT + KBY supplementation may ameliorate skin capillary damage. Both JHT and KBY contain herbs with ku-oketsu effects [18, 20]. Therefore, considering the alleviation of blood flow abnormalities in rosacea after the administration of JHT and KBY, it is not necessary to prescribe JHT and KBY simultaneously.

As an antioxidative drug, anti-oketsu Kampo medicines not only relieved capillary tension but also capillary contraction under conditions such as oxidation acceleration. Therefore, Kampo medicines are thought to regulate oxidative stress and harmonize biological functions

[23].

Increased production of reactive oxygen species (ROS) by innate immune cells such as cutaneous neutrophils is closely associated with symptoms of rosacea. Therefore, the suppression of ROS production by azelaic acid or metronidazole is considered effective against rosacea [24]. Rosacea purpura is an erythematous inflammatory disease that occurs when blood extravasates into the skin from the capillaries. ROS is one of the main factors that cause vascular injury.

In Japan, there are two types of JHT, which include cherry bark and oak bark, and the difference between the effectiveness of JHTs prepared using different methods has been examined in various studies. In the present study, JHT containing cherry bark was used. The antioxidant ability of oak bark alone is known to be slightly superior to that of cherry bark. However, when the bark extracts were prepared using the brewing method and mixed with other drugs, the antioxidative effect of cherry bark was superior to that of oak bark. Overall, under clinical conditions, a single crude drug may not show effectiveness, but the synergistic effect of a combination of cherry and oak barks mixed with other herbs such as Glycyrrhiza and Bupleurum roots, which have strong antioxidative, anti-inflammatory, and detoxification abilities, can be observed. The antioxidant effects of KBY have been previously reported [23]. JHT and KBY might have induced a reduction in purpura area by inhibiting oxidative stress

in skin capillaries. Our results indicate that the use of KBY or JHT alone was not sufficient to cure cutaneous purpura, whereas a combination of JHT and KBY can be expected to completely clear cutaneous purpura, which may be because of the synergistic effects of JHT and KBY.

Skin atrophy is the most common adverse effect of long-term steroid use and can cause steroid-induced rosacea [25]. Our present results provide considerable evidence of JHT + KBY's efficacy against steroid-induced rosacea.

JHT is thought to repair skin damage via its anti-inflammatory action [26,27]. However, JHT alone was insufficient to treat skin thinning, whereas the recovery from skin thinning was prominent in the JHT + KBY group. Therefore, the water utilization-enhancing effects of ku-oketsu herbs are more important than their anti-inflammatory effects. *Poria sclerotium* is a component of JHT, which has a strong water utilization-enhancing effect and is good for reducing edema all over the body. The synergistic anti-inflammatory effects of ku-oketsu and ri-sui (regulatory effect on body fluids) in Kampo medicine include expulsion of excess fluid from the body via promotion of diuretic, perspiratory, and warming effects and by metabolic acceleration, which result in transportation of retained fluid from the stomach to the bowels. The anti-inflammatory, ku-oketsu, and ri-sui effects are necessary remedies to cure skin diseases. We believe that the effectiveness of the ku-oketsu and ri-sui effects is higher than

that of the anti-inflammatory or synergistic effects of ku-oketsu and ri-sui in terms of the repair of thinning skin.

The results of fecal metabolomic analysis might have been influenced by the intake of Kampo medicines. Intestinal bacterial metabolism plays an important role in promoting the medicinal effects of Kampo. Additionally, the composition of intestinal microbiota is altered by the intake of Kampo medicines [28]. Spermine is a polyamine involved in cellular metabolism, and the gut microbiota is considered one of the most important sources of polyamines in the human body. We hypothesized that the increase in fecal spermine levels in the JHY + KBY group was because of the effect of the herbs on the gut microbiota. We believe that this hypothesis is validated by the results showing a decrease in S-adenosylmethionine levels in the JHT + KBY group, because S-adenosylmethionine is an important intermediate in polyamine metabolism that is finally metabolized to spermine. Polyamines, including spermine, have an anti-inflammatory effect by inhibiting macrophage activation. However, since no increase in polyamine levels other than spermine was observed in the JHT+KBY group in the present study, it cannot be determined whether the anti-inflammatory effects of polyamines are responsible for the improvement of rosacea-like symptoms.

Regarding the relationship between the intestinal microflora and skin health, *Lactobacillales* are involved in maintenance of skin health [29]. In our previous study, the

relative abundance of lactobacilli in the KBY inoculation group increased [22], suggesting that increased fecal spermine levels are associated with an improvement in rosacea-like symptoms.

In the JHT + KBY group, malic acid levels significantly decreased, whereas Asn levels increased. This may be due to an alteration in the malate–aspartate shuttle, resulting in a decrease in malate levels and an increase in levels of Asn, which is biosynthesized from aspartate. However, its association with alleviation of rosacea symptoms remains unclear. Moreover, we are uncertain regarding the reason for the increase in uracil levels in the JHT + KBY group.

Interestingly, the levels of hydroxyproline, a major component of collagen, markedly increased ($p = 0.051$) in the JHT + KBY group. Hydroxyproline is thought to be useful for recovery from skin damage and thinning induced by long-term corticosteroid use. As estimated by amino acid analysis, the hydroxyproline contents in the diets used in this experiment were 80 mg/100 g for the standard diet (MF diet), 81.875 mg/100 g for the JHT group, and 82.15 mg/100 g for the JHT + KBY group, which revealed no significant differences in the hydroxyproline contents. Therefore, the increase in hydroxyproline levels in the JHT + KBY group could not be attributed to the orally ingested herbal medicine. During the recovery from skin damage, collagen is synthesized, and the amount of

hydroxyproline increases. We believe that the increase in fecal hydroxyproline levels in the JHT + KBY group was associated with the accelerated recovery from rosacea-like skin damage caused by intake of Kampo medicine.

In conclusion, Kampo medicine comprises a variety of herbal medicines that have synergistic effects. Rosacea has many clinical manifestations, including inflammation and vascular abnormalities, which cannot be addressed using a single-drug regimen. In clinical cases of rosacea, seinetsu and anti-oketsu drugs are often prescribed in Kampo medicine [30]. In this study, JHT was the seinetsu formula, and KBY was the anti-oketsu formula. However, the mechanism by which these Kampo medicines exert their effects remains unknown. This study demonstrated that anti-oketsu and seinetsu agents play a role in the healing of rosacea-like symptoms.

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CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interests for this article.

380 APPROVAL OF THE RESEARCH PROTOCOL BY
381 AN INSTITUTIONAL REVIEWER BOARD AND
382 THE APPROVAL NUMBER

383 This study was approved by the Animal Care and Use Committee of Hyogo Medical
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385
386 INFORMED CONSENT

387 N/A

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390 THE STUDY/TRIAL

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393 ANIMAL STUDIES

394 All protocols in this study were approved by the Animal Care and Use Committee of Hyogo
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396 Regulations of Hyogo Medical University.

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Figure legends

Fig 1. The experimental timeline of this study. Clobetasol propionate ointment was applied
on days 0–9. The mice were fed Kampo-containing diet on days 9–37. Capillary blood flow
velocity and skin purpura were observed on days 0–37.

**Fig 2. Chronological changes in the average blood flow velocities in skin capillaries of each
group.** The blood flow velocities of the JHT (N = 9) and JHT + KBY (N = 10) groups were
significantly higher than that of the no-Kampo group (N = 9). Moreover, blood flow velocity

of the no-Kampo group was significantly lower than that observed in the control group (N = 5). The average blood flow velocities in the JHT + KBY group were significantly higher than those in the no-Kampo group on individual days from days 21 to 30, and the blood flow velocity in the JHT group was significantly higher than that in the no-Kampo group on day 37. Data represent the means \pm standard errors of the means (SEMs). **,## p < 0.01; *,# p < 0.05.

JHT: 10 days of steroid application followed by JHT-containing feed

JHT+KBY: 10 days of steroid application followed by JHT + KBY feed

no-Kampo: 10 days of steroid application followed by standard MF diet

control: No steroid, standard MF diet

Fig 3. Chronological changes in the average areas of the skin purpura in different experimental groups. (A) Average area of skin purpura in the no-Kampo group (N = 9) was significantly higher than those in the other groups. After day 16, the mean purpura area in the JHT + KBY group was significantly smaller than that in the no-Kampo and JHT group. Data represent the means \pm SEMs. **,##,†† p < 0.01; *,#,† p < 0.05.

(B) Typical skin purpura on days 0, 9 and 37. The area of purpura was determined by binarising with an automatic threshold in imageJ software and measuring the black area. Dark

prominent purpura was observed on day 9 (after continuous steroid application) and on day 37 in the no-kampo group (white arrows). On the other hand, the JHT group had smaller purpura on day 37 and the JHT+KBY group had no darker purpura, similar to day 0. Bar= 200 μ m

JHT: 10 days of steroid application followed by JHT-containing feed

JHT+KBY: 10 days of steroid application followed by JHT + KBY feed

no-Kampo: 10 days of steroid application followed by standard MF diet

control: No steroid, standard MF diet

Fig 4. Histological images (hematoxylin and eosin [HE] staining) of dorsal skin samples. On day 9, the no-Kampo group showed horny layer peeling and thinning of the granular layer (A, arrows). On day 37, sufficient recovery of skin abnormalities was not observed in the no-Kampo group (B, arrows), whereas the JHT + KBY group showed an almost normal epidermis and granular layer (C, arrows). Recovery in the JHT alone group was not sufficient on day 37 (D, arrows). Bar = 100 μ m.

JHT: 10 days of steroid application followed by JHT-containing feed

JHT+KBY: 10 days of steroid application followed by JHT + KBY feed

no-Kampo: 10 days of steroid application followed by standard MF diet

524 control: No steroid, standard MF diet

525

526 **Table.1 The list of ingredients for JHT.**

527

528 **Table.2 The list of ingredients for KBY.**

529

530 **Table 3. Results of metabolomic analysis.** Substances whose contents differed between the

531 JHT + KBY and no-Kampo groups at a significance level of less than 0.2 are shown.

532 *, $p < 0.05$; +, $p < 0.10$.

533 JHT+KBY: 10 days of steroid application followed by JHT + KBY feed

534 no-Kampo: 10 days of steroid application followed by standard MF diet

535

536 **Table 4. Summary of Kampo effects.** ◎: recovery to control level; ○: recovery, but not to

537 control level; ×: no difference from no-kampo group results.

Fig1

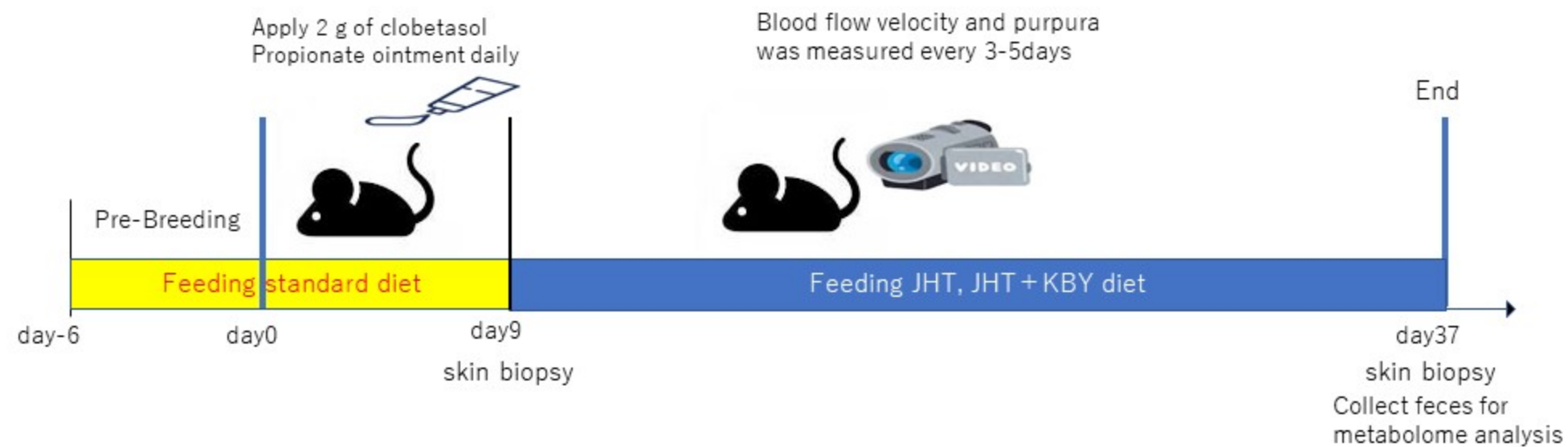
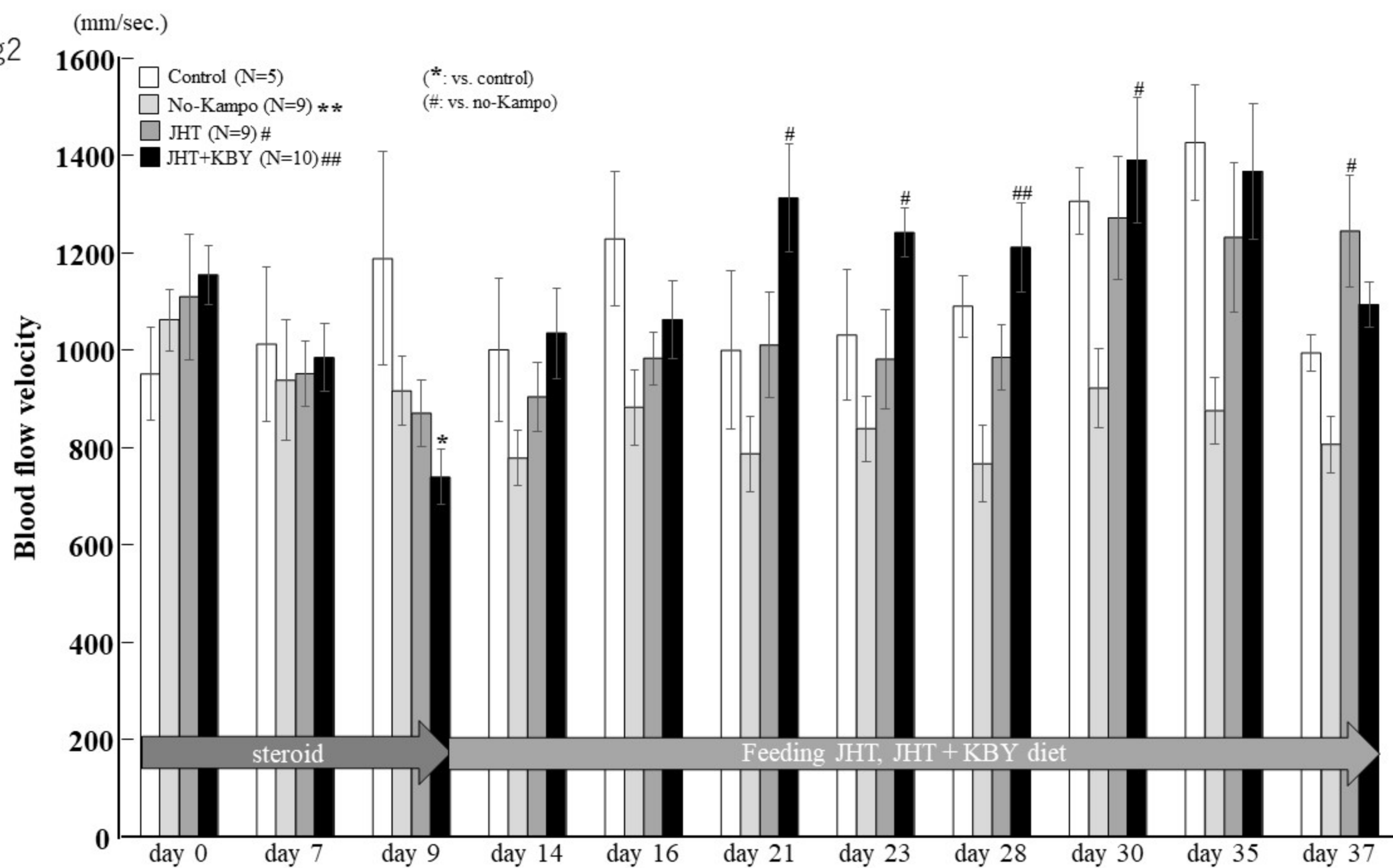


Fig2



A(mm²)**18**

□ Control (N=5)

▒ No-Kampo (N=9) **

▓ JHT (N=9) **, #

■ JHT+KBY (N=10) ##, †

(*: vs. control)

(#: vs. no-Kampo)

(†: vs. JHT)

16**14****12****10****8****6****4****2****0**

Area of Purpura

day0

day7

day9

day14

day16

day21

day23

day28

day30

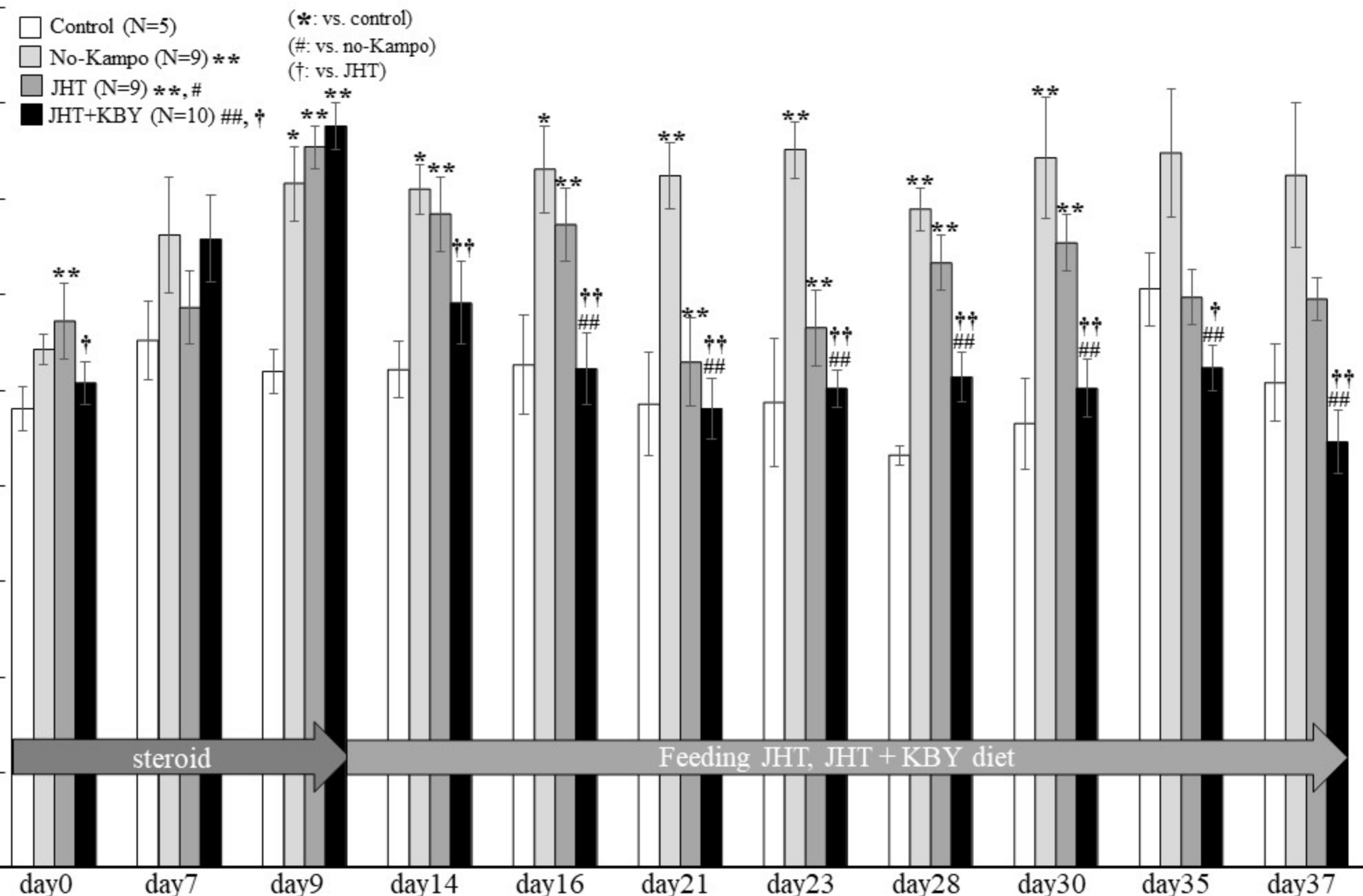
day35

day37

steroid

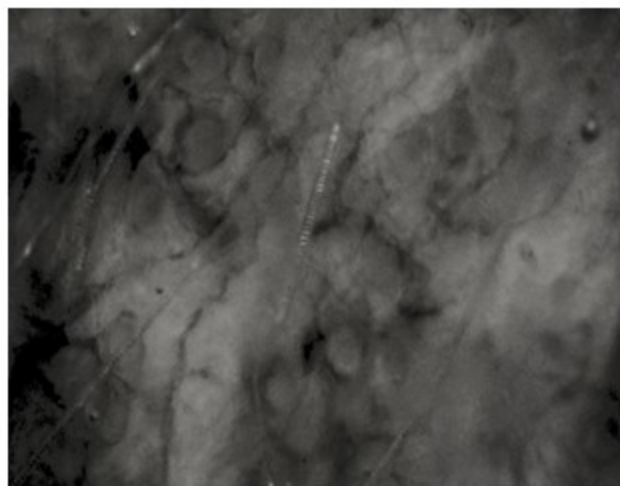
Feeding JHT, JHT + KBY diet

Fig3

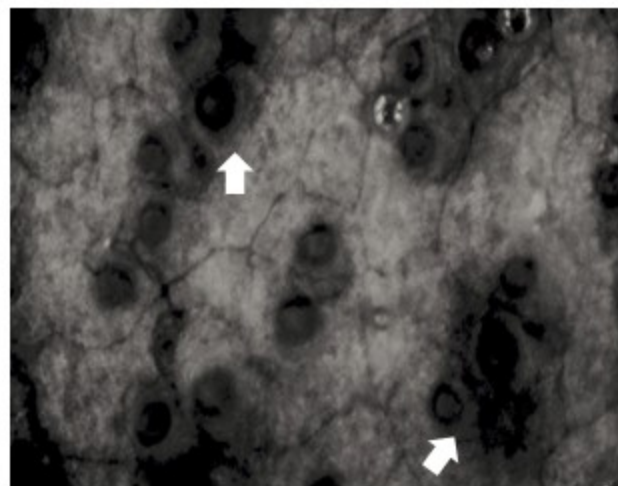


B

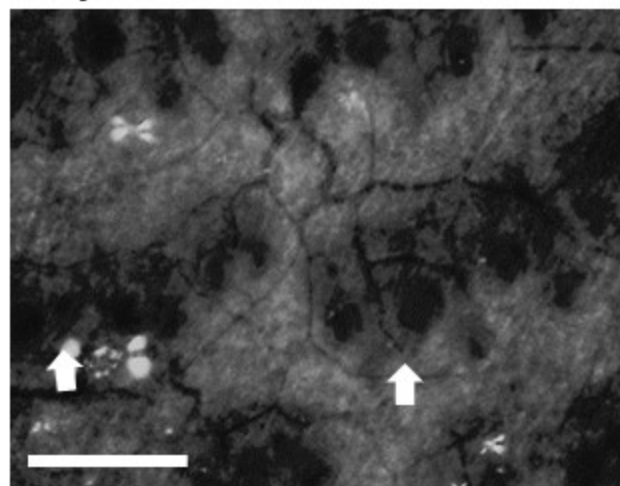
Fig3



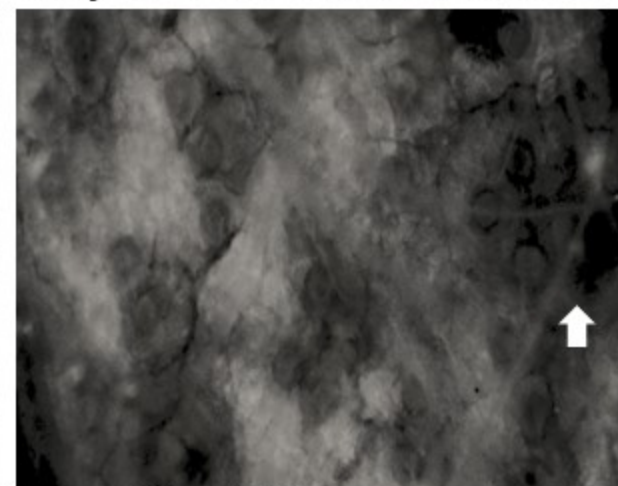
Day 0, Control



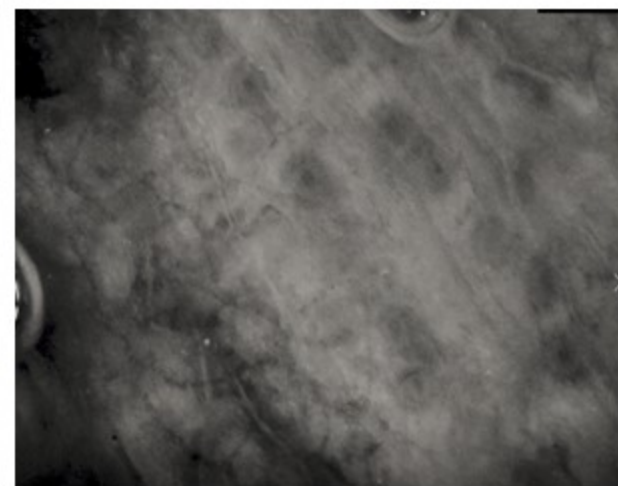
Day 9, after steroid treatment



Day 37, no-Kampo



Day 37, JHT



Day 37, JHT + KBY

Fig4

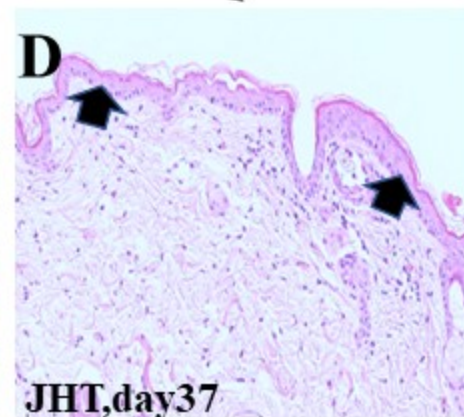
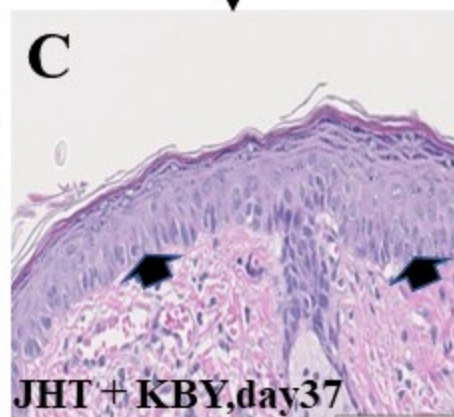
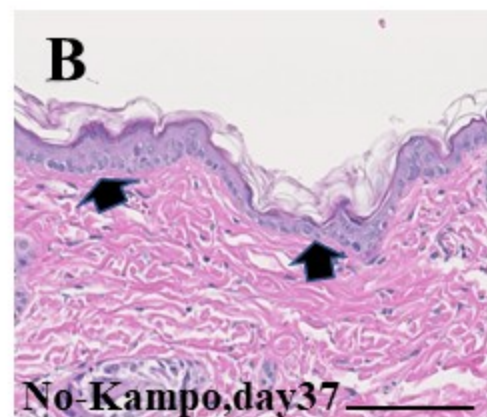
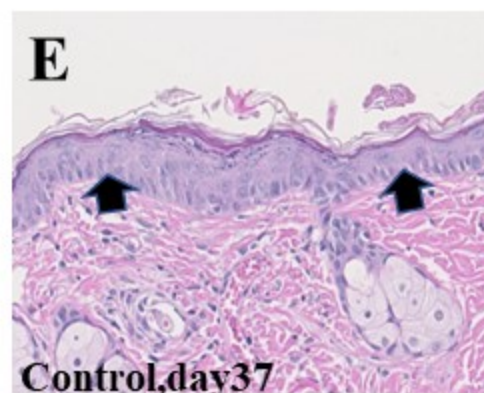
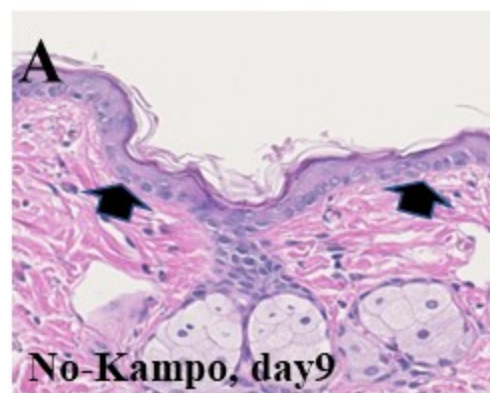


Table1

Ingredients for Jumi-Haidokuto		
Saiko	root of <i>Bupleurum falcatum</i>	2.5g
Kikyo	root of <i>Platycodon grandiflorus</i>	2.5g
Senkyu	rhizome of <i>Cnidium officinale</i>	2.5g
Bukuryo	sclerotia of <i>Wolfiporia cocos</i>	2.5g
Bofu	root of <i>Saposhnikovia divaricata</i>	2.5g
Syokyo	rhizome of <i>Zingiber officinale Roscoe</i>	1.0g
Keigai	spike of <i>Schizonepeta tenuifolia</i>	1.5g
Dokkatsu	rhizome of <i>Aralia cordata rhizome</i>	1.5g
Ohi	bark of <i>Cerasus jamasakura</i>	2.5g
Kanzo	<i>Glycyrrhiza uralensis</i>	1.5g

Table2

Ingredients for Keishi-Bukuryo-gan-ka-Yokuinin		
Keihi	bark of <i>Cinnamomum cassia</i>	4.0g
Shakuyaku	root of <i>Paeonia lactiflora</i>	4.0g
Tounin	kernel of <i>Prunus persica</i>	4.0g
Bukuryo	sclerotia of <i>Wolfiporia cocos</i>	4.0g
Botanpi	bark of <i>Paeonia suffruticosa</i>	3.0g
Yokuinin	seed of <i>Coix lacryma-jobi var. ma-yuen</i>	10.0g

Table3

Compound name	Concentration (nmol/g)		JHT+KBY vs no-Kampo		
	JHT+KBY	no-Kampo	Ratio	p-value	
Spermine	6.641 ± 0.609	4.152 ± 1.077	1.599	0.013	*
Malic acid	631.376 ± 265.202	1109.409 ± 287.566	0.569	0.026	*
Uracil	1115.592 ± 390.104	1788.622 ± 472.566	0.624	0.041	*
Hydroxyproline	141.508 ± 58.951	69.642 ± 27.777	2.032	0.051	+
Asn	3112.118 ± 1509.778	1556.939 ± 880.899	1.999	0.091	+
S-Adenosylmethionine	7.715 ± 4.727	19.115 ± 7.450	0.404	0.097	+
Trp	882.913 ± 252.073	624.920 ± 191.431	1.413	0.109	
Cytidine	280.466 ± 163.780	133.740 ± 67.285	2.097	0.120	
Choline	272.884 ± 76.452	429.653 ± 183.914	0.635	0.135	
Lactic acid	3939.503 ± 3895.985	7456.559 ± 2984.611	0.528	0.150	
Fructose 6-phosphate	49.474 ± 11.452	24.580 ± 5.915	2.013	0.159	
Tyr	6971.549 ± 2364.243	5071.624 ± 1291.861	1.375	0.164	
Ile	5321.530 ± 1897.113	3931.509 ± 984.332	1.354	0.196	
Guanosine	675.046 ± 471.164	331.191 ± 238.572	2.038	0.196	
Betaine aldehyde_+H ₂ O	2.496 ± 0.939	3.180 ± 0.488	0.785	0.199	

Table4

	blood flow velocity	skin purpura	skin thinning
KBY [23]	⊙	○	⊙
JHT	⊙	○	×
JHT+KBY	⊙	⊙	⊙