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Effect of shinbuto and ninjinto on prostaglandin E$_2$ production in lipopolysaccharide-treated human gingival fibroblasts

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Previously, we revealed that several kampo medicines which are used for the patients with excess and/or medium patterns [kakkonto (TJ-1), shosaikoto (TJ-9), hangeshashinto (TJ-14), and orento (TJ-120)] decreased prostaglandin (PG)E$_2$ by LPS-treated human gingival fibroblasts (HGFs). Currently, we examined other kampo medicines which are used for the patients with deficiency pattern [bakumondoto (TJ-29), shinbuto (TJ-30), ninjinto (TJ-32), and hochuekkito (TJ-41)] and the herbs which construct shinbuto and ninjinto using the same experimental model. Shinbuto and ninjinto concentration-dependently decreased LPS-induced PGE$_2$ production by HGFs, whereas hochuekkito weakly decreased and bakumondoto did not decrease PGE$_2$ production. Shinbuto and ninjinto did not alter cyclooxygenase (COX) activities and the expressions of molecules involved in arachidonic acid cascade. Next, we examined which herbs constructing shinbuto and ninjinto decrease LPS-induced PGE$_2$ production. Among these herbs, shokyo (Zingiberis Rhizoma) and kankyo (Zingiberis Processum Rhizoma) strongly and concentration-dependently decreased LPS-induced PGE$_2$ production. However, both shokyo and kankyo did not alter the expressions of molecules involved in arachidonic acid cascade. These results suggest that shokyo and kankyo suppress phospholipase (PL)A$_2$ activity. We demonstrated that kampo medicines for the patients with deficiency pattern may suppress inflammatory responses in addition to those with excess and medium patterns. Moreover, kampo medicines which contain shokyo or kankyo are considered to be effective for the treatment of the inflammatory diseases.
Effect of shinbuto and ninjinto on prostaglandin E₂ production in lipopolysaccharide-treated human gingival fibroblasts

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ABSTRACT

Previously, we revealed that several kampo medicines which are used for the patients with excess and/or medium patterns [kakkonto (TJ-1), shosaikoto (TJ-9), hangeshashinto (TJ-14), and orento (TJ-120)] decreased prostaglandin (PG)E₂ by LPS-treated human gingival fibroblasts (HGFs). Currently, we examined other kampo medicines which are used for the patients with deficiency pattern [bakumondoto (TJ-29), shinbuto (TJ-30), ninjinto (TJ-32), and hochuekkito (TJ-41)] and the herbs which construct shinbuto and ninjinto using the same experimental model. Shinbuto and ninjinto concentration-dependently decreased LPS-induced PGE₂ production by HGFs, whereas hochuekkito weakly decreased and bakumondoto did not decrease PGE₂ production. Shinbuto and ninjinto did not alter cyclooxygenase (COX) activities and the expressions of molecules involved in arachidonic acid cascade. Next, we examined which herbs constructing shinbuto and ninjinto decrease LPS-induced PGE₂ production. Among these herbs, shokyo (Zingiberis Rhizoma) and kankyo (Zingiberis Processum Rhizoma) strongly and concentration-dependently decreased LPS-induced PGE₂ production. However, both shokyo and kankyo did not alter the expressions of molecules involved in arachidonic acid cascade. These results suggest that shokyo and kankyo suppress phospholipase (PL)A₂ activity. We demonstrated that kampo medicines for the patients with deficiency pattern may suppress inflammatory responses in addition to those with excess and medium patterns. Moreover, kampo medicines which contain shokyo or kankyo are considered to be effective for the treatment of the inflammatory diseases.

INTRODUCTION

Periodontal disease is an inflammation disease of the gingiva and destroy periodontal tissues. In severe case, alveolar bone is absorbed. In inflammatory responses and tissue degradation, prostaglandin E₂ (PGE₂), interleukin (IL)-6, and IL-8 play important roles. Because PGE₂ has several functions in vasodilation, the enhancement of vascular permeability and pain, and osteoclastogenesis induction, PGE₂ participate in inflammatory responses and alveolar bone resorption in periodontal disease (Noguchi and Ishikawa,
Previously, we reported that several kampo medicines, shosaikoto (TJ-9) (Ara et al., 2008b), orento (TJ-120) (Ara et al., 2010), hangeshashinto (TJ-14) (Nakazono et al., 2010), and kakkonto (TJ-1) (Kitamura et al., 2014), suppress lipopolysaccharide (LPS)-induced PGE$_2$ production by human gingival fibroblasts (HGFs). Moreover, we showed shokyo, kanzo, and keihi, which are herbs contained in kakkonto, decrease PGE$_2$ production (Ara and Sogawa, 2016). These results suggested that these kampo medicines and herbs have anti-inflammatory effects in periodontal disease.

However, these kampo medicines are used for the patients with excess pattern and medium pattern. The kampo medicines for those with deficiency pattern remain to be elucidated. In the present study, therefore, we examined the anti-inflammatory effects of the kampo medicines for that with deficiency pattern [bakumondoto (TJ-29), shinbuto (TJ-30), ninjinto (TJ-32), and hochuekkito (TJ-41)], which are used for the treatment of inflammatory diseases. Moreover, we examined the effect on PGE$_2$ production using herbs constitute the kampo medicines which decrease PGE$_2$ production.

**MATERIALS AND METHODS**

**Reagents**

Kampo medicines (bakumondoto, shinbuto, ninjinto, and hochuekkito) were purchased from Tsumura & Co. (Tokyo, Japan). Powders of 8 herbs (bukuryo, bushi, kankyo, kanzo, ninjin, shakuyaku, shokyo, and sojutsu) were provided by Tsumura & Co. The ingredients of shinbuto and ninjinto formula are shown in Tables 1 and 2. Powders of kampo medicines or herbs were suspended in Dulbecco’s modified Eagle’s medium (D-MEM, Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (culture medium), and were rotated at 4°C overnight. Then, the suspensions were centrifuged and the supernatants were filtrated through a 0.45 μm-pore membrane. Lipopolysaccharide (LPS) from *Porphyromonas gingivalis* 381 was provided by Professor Nobuhiro Hanada (School of Dental Medicine, Tsurumi University, Japan). Arachidonic acid was purchased from Cayman Chemical (Ann Arbor, MI). Other reagents were purchased from Nacalai tesque (Kyoto, Japan).

**Cells**

HGFs were prepared as described previously (Nakazono et al., 2010). In brief, HGFs were prepared from free gingiva during the extraction of an impacted tooth with the informed consent of the subjects who consulted Matsumoto Dental University Hospital. The free gingival tissues were cut into pieces and seeded onto 24-well plates (AGC Techno Glass Co., Chiba, Japan). HGFs were maintained in culture medium at 37°C in a humidified atmosphere of 5% CO$_2$. For passage, HGFs were trypsinazed, suspended, and plated into new cultures in a 1:3 dilution ratio. HGFs were used between the 10th to 15th passages in the assays. This study was approved by the Ethical Committee of Matsumoto Dental University (No. 0063).
Measurement of cell viability
The numbers of cells were measured using WST-8 (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. In brief, the media were removed by aspiration and the cells were treated with 100 µl of mixture of WST-8 with culture medium for 2 h at 37°C in CO₂ incubator. Optical density was measured (measured wavelength at 450 nm and reference wavelength at 655 nm) using an iMark microplate reader (Bio-Rad, Hercules, CA), and the mean background value was subtracted from each value. Data is represented as means ± S.D. (n = 4).

Measurement of prostaglandin E (PGE₂), interleukin (IL)-6, and IL-8
HGFs were seeded in 96-well plates (10,000 cells/well) and incubated in serum-containing medium at 37°C overnight. Then, the cells were treated with various concentrations of each kampo medicine (0, 0.01, 0.1, and 1 mg/ml) or each herb (0, 10, 30, and 100 µg/ml) in the absence or presence of LPS (10 ng/ml) for 24 h (200 µl each well) in triplicate or quadruplicate for each sample. After the culture supernatants were collected, viable cell numbers were measured using WST-8 as described above.

The concentrations of PGE₂, IL-6, and IL-8 in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (PGE₂, Cayman Chemical; IL-6 and IL-8, Thermo Fisher Scientific Inc., Camarillo, MA, USA), and were adjusted by the number of viable cells. Data are represented as pg per 10,000 cells (mean ± S.D.).

Measurement of cyclooxygenase (COX)-2 activity
COX-2 activity was evaluated as shown previously (Wilborn et al., 1995) with slight modification. In brief, to estimate COX-2 activity, HGFs were treated with LPS and herb for 8 h, washed, and incubated in culture medium containing exogenous arachidonic acid (10 µM). The concentrations of PGE₂ in the supernatants were measured by ELISA. Data are represented as pg per 10,000 cells (mean ± S.D.).

Preparation of cell lysates
HGFs were cultured in 60-mm dishes and treated with combinations of LPS and herb for the indicated times. Then, cells were washed twice with Tris-buffered saline, transferred into microcentrifuge tubes, and centrifuged at 6,000 × g for 5 min at 4°C. Supernatants were aspirated and cells were lysed on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethyleneglycol bis(2-aminoethyl)ether)tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 5 µg/ml leupeptin, and 1 µg/ml pepstatin) for 30 min at 4°C. Then, samples were centrifuged at 12,000 × g for 15 min at 4°C, and supernatants were collected. The protein concentration was measured using a BCA Protein Assay Reagent kit (Pierce Chemical Co., Rockford, IL).

Western blotting
The samples (10 µg of protein) were fractionated in a polyacrylamide gel under reducing conditions and transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P; GE Healthcare, Uppsala, Sweden). The membranes were blocked with 5% ovalbumin for 1 h at room temperature and incubated with primary antibody for an additional
The membranes were further incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized with an ECL kit (GE Healthcare).

Antibodies against COX-2 (sc-1745, 1:500 dilution), cytosolic PLA$_2$ (cPLA$_2$) (sc-438, 1:200 dilution), annexin 1 (sc-11387, 1:1,000 dilution), and actin (sc-1616, 1:1,000 dilution), which detects a broad range of actin isoforms, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against extracellular signal-regulated kinase (ERK; p44/42 MAP kinase antibody, 1:1,000 dilution) and phosphorylated ERK [Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) monoclonal antibody, 1:2,000 dilution] were from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-goat IgG (sc-2020, 1:20,000 dilution) was from Santa Cruz, and anti-rabbit IgG (1:20,000 dilution) and anti-mouse IgG (1:20,000 dilution) were from Dako-Cytomation (Glostrup, Denmark).

Statistical analysis
Differences between groups were evaluated by the two-tailed pairwise comparison test with a pooled variance, followed by correction with the Holm method (total 10 null hypotheses; 5 null hypotheses without kampo vs. with kampo in the absence or presence of LPS in Fig. 1, total 10 null hypotheses; 3 null hypotheses without kampo vs. with kampo in the absence of LPS, 3 null hypotheses without kampo vs. with kampo in the presence of LPS, and 4 null hypotheses without LPS vs. with LPS in Fig. 2). Differences between the control group and experimental groups were evaluated by a two-tailed Dunnett’s test (Figs. 3 and 6).

All computations were performed with the statistical program R (http://www.r-project.org/). Dunnett’s test was performed using the ‘glht’ function in the ‘multcomp’ package. Values with $P < 0.05$ were considered significantly different.

RESULTS

Effects of kampo medicines on HGFs viability
First, we examined the effect of four kampo medicines (bakumondoto, shinbuto, ninjinto, and hochuekkito) on HGFs viability. Bakumondoto did not affect the viability up to 10 mg/ml at 24 h treatment (Fig. 1). In contrast, Shinbuto, ninjinto, and hochuekkito did not affect the viability up to 2 mg/ml but decreased at 5 mg/ml and 10 mg/ml (Fig. 1). Therefore, up to 1 mg/ml of kampo medicines was used in further experiments because we used the same concentration of kampo medicines in previous studies (Ara et al., 2008b, 2010; Nakazono et al., 2010; Kitamura et al., 2014).

Effects of kampo medicines on prostaglandin (PG)E$_2$, interleukin (IL)-6, and IL-8 production
We examined whether these kampo medicines affect the production of PGE$_2$ and inflammatory cytokines (IL-6 and IL-8) by HGFs. The concentrations of PGE$_2$, IL-6, and IL-8 were adjusted according to viable cell number. HGFs treated with 10 ng/ml of LPS produced large amounts of PGE$_2$, IL-6, and IL-8. Shinbuto and ninjinto strongly and concentration-dependently decreased LPS-induced PGE$_2$ production (Fig. 2). In contrast, bakumondoto and hochuekkito had no or little effect on PGE$_2$ production.
Bakumondoto weakly and shinbuto, ninjinto, and hochuekkito strongly increased LPS-induced IL-6 production (Fig. 2). Bakumondoto and hochuekkito weakly increased LPS-induced IL-8 production, but shinbuto and ninjinto did not affect IL-8 production (Fig. 2).

From these results, we selected two kampo medicines (shinbuto and ninjinto which decreased PGE$_2$ production) and used in following experiments.

**Effects of shinbuto and ninjinto on arachidonic acid cascade**

To clarify the mechanism that shinbuto and ninjinto decreased LPS-induced PGE$_2$ production more directly, we examined the effects of these two kampo medicines on arachidonic acid cascade. First, we examined the effect of shinbuto and ninjinto on COX activity. In order to bypass PLA$_2$, we added exogenous arachidonic acid to HGFs treated with LPS alone or LPS plus kampo medicine (shinbuto or ninjinto). Then, we measured PGE$_2$ level produced by COX. However, shinbuto and ninjinto did not affect LPS-induced PGE$_2$ production (Fig. 3).

Next, we examined whether shinbuto and ninjinto affect the expression of molecules in the arachidonic acid cascade. $c$PLA$_2$, which is the most upstream enzyme in the arachidonic acid cascade, and releases arachidonic acid from plasma membranes. Shinbuto and ninjinto did not alter $c$PLA$_2$ expression in the absence or presence of LPS (Fig. 4A). COX-2 was weakly expressed in the absence of LPS, and the treatment with LPS alone increased COX-2 expression. However, shokyo did not alter but kankyo slightly increased LPS-induced COX-2 expression (Fig. 4). Annexin1 (also named lipocortin1) is produced by glucocorticoids and inhibits $c$PLA$_2$ activity (Gupta et al., 1984; Wallner et al., 1986). Shokyo and kankyo slightly increased annexin1 expression (Fig. 4A) in a concentration-dependent manner (Fig. 4B).

At last, we examined the effects of shinbuto and ninjinto on ERK phosphorylation. $c$PLA$_2$ is directly phosphorylated and activated by phosphorylated ERK (Lin et al., 1993; Gijón et al., 1999). Therefore, we examined whether shinbuto and ninjinto suppress LPS-induced ERK phosphorylation. LPS treatment enhanced ERK phosphorylation at 0.5 h and then its phosphorylation was attenuated. However, 1 mg/ml of shinbuto and ninjinto did not affect LPS-induced ERK phosphorylation (Fig. 5).

**Effects of herbs on PGE$_2$ production and molecular expression in the arachidonic acid cascade**

We examined whether herbs which construct shinbuto and ninjinto affect LPS-induced PGE$_2$ production by HGFs. When HGFs cells were treated with 10 ng/ml of LPS, HGFs cells produced large amounts of PGE$_2$. Bukuryo increased LPS-induced PGE$_2$ production. Shokyo, kankyo and kanzo strongly and significantly decreased LPS-induced PGE$_2$ production (Fig. 6A). Moreover, shokyo and kankyo decreased PGE$_2$ production in a concentration-dependent manner (Fig. 6B). Other herbs had no or little effect on PGE$_2$ production.

Next, we examined whether shokyo and kankyo affect the expression of molecules in the arachidonic acid cascade. Both shokyo and kankyo did not affect the expression of $c$PLA$_2$, annexin1, and COX-2 (Fig. 7).
DISCUSSION

At first, we explain the importance of the usage of HGFs in the study of periodontal disease as described in our previous studies (Kamemoto et al., 2009; Ara et al., 2010; Nakazono et al., 2010; Ara et al., 2012; Kitamura et al., 2014; Ara and Sogawa, 2016). In periodontal tissue, HGFs are the most prominent cells. Moreover, LPS-treated HGFs produce inflammatory chemical mediators such as PGE₂ and inflammatory cytokines such as IL-6 and IL-8 (Sismey-Durrant and Hopps, 1991; Bartold and Haynes, 1991; Tamura et al., 1992). Moreover, HGFs sustained to produce PGE₂ (Ara et al., 2008a), IL-6, and IL-8 (Ara et al., 2009) in the presence of LPS. Therefore, the large amount of chemical mediators and cytokines derived from HGFs may be contained in periodontal tissues. From these findings, we believe that examining the effects of drugs on HGFs is needed in the study of periodontal disease.

In the present study, we examined the effect of kampo medicines for the patients with deficiency pattern on LPS-induced PGE₂, IL-6, and IL-8 production by HGFs. Shinbuto and ninjinto dose-dependently decreased LPS-induced PGE₂ production (Fig. 2) similar to shosaikoto, hangeshashinto, orento, and kakkonto (Ara et al., 2008b; Nakazono et al., 2010; Ara et al., 2010; Kitamura et al., 2014). However, shinbuto and ninjinto increased LPS-induced IL-6 and IL-8 productions (Fig. 2). In general, acid non-steroidal anti-inflammatory drugs (NSAIDs) show anti-inflammatory effects by suppression of PGE₂ production, even though they do not affect IL-6 and IL-8 production. Therefore, sour results suggest that shinbuto and ninjinto show anti-inflammatory effects in periodontal disease as well as acid NSAIDs.

In the experiments at herb level, shokyo (Zingiberis Rhizoma), kankyo (Zingiberis Processum Rhizoma), and kanzo (Glycyrrhizae Radix) decreased PGE₂ production (Fig. 6). Shokyo is contained in shinbuto (Table 1) and kankyo and kanzo are contained in ninjinto (Table 2). Shokyo is the powdered rhizome of ginger (Zingiber officinale Roscoe), whereas, kankyo is the steamed and powdered rhizome of ginger. Many reports showed that ginger has anti-inflammatory effects in human (Afzal et al., 2001; Lakhan et al., 2015), animal models (Thomson et al., 2002; Aimbire et al., 2007; El-Abhar et al., 2008), and in vitro models (Ara and Sogawa, 2016; Podlogar and Verspohl, 2012). Shokyo contains gingerols such as 6-, 8-, and 10-gingerols. With prolonged strage or heat-treatment of ginger, gingerols are converted to shogaols, which are the dehydrated form of the gingerols (Afzal et al., 2001). Therefore, kankyo contains the large amount of shogaols.

In the recent study, we showed that shokyo suppressed LPS-induced PGE₂ production by HGFs and that shokyo may suppress PLA₂ activity (Ara and Sogawa, 2016). In the present study, we examined the effect of kankyo in comparison with shokyo. We showed that shokyo and kankyo only slightly increase cPLA₂ expression but did not alter annexin 1 expression (Fig. 7). Moreover, we showed that shinbuto and ninjinto, which contain shokyo and kankyo respectively, did not altered PGE₂ production when arachidonic acid was added to bypass their upstream pathway (Fig. 3). These data suggest that shokyo and kankyo did not affect the downstream pathway of arachidonic acid, which include COX-2 and PGE synthase. In addition, shinbuto and ninjinto did not affect ERK phosphorylation (Fig. 5). From our findings described above could not explain the mechanism to decrease PGE₂ production. Because gingerols in ginger...
are reported to inhibit both calcium-independent PLA$_2$ (iPLA$_2$) and cPLA$_2$ activities (Nievergelt et al., 2011), shokyo and kankyo are suggested to inhibit PLA$_2$ as discussed in previous study (Ara and Sogawa, 2016). Previously, we showed that cPLA$_2$ is the main isoform in HGFs (Ara and Sogawa, 2016) among the subtypes such as cPLA$_2$, iPLA$_2$, and secretory PLA$_2$ (sPLA$_2$) (Burke and Dennis, 2009). Therefore, shokyo and kankyo may mainly inhibit cPLA$_2$ activity in HGFs. Previously, we reported that orento decreases LPS-induced PGE$_2$ production via the suppression of ERK phosphorylation (Ara et al., 2010). However, orento also may decrease LPS-induced PGE$_2$ production by inhibition of cPLA$_2$ activity because orento contains kankyo.

We demonstrated that shokyo and kankyo concentration-dependently decreased LPS-induced PGE$_2$ production (Fig. 6A), and that the effect of kankyo is slightly strong compared to that of shokyo (Fig. 6B). Previous study showed that 6- and 8-gingerols did not inhibit cPLA$_2$ activity, but 10-gingerol and 6-, 8-, and 10-shogaols inhibit cPLA$_2$ activity (Nievergelt et al., 2011). Therefore, the difference in this effect on PGE$_2$ production between shokyo and kankyo may be due to the amount of shogaols in these herbs.

We demonstrated that shinbuto and ninjinto slightly increased annexin1 expression (Fig. 4). However, the involvement of annexin1 in decreased PGE$_2$ production is unlikely. Shokyo and kankyo did not alter annexin1 expression (Fig. 7). All 4 herbs other than shokyo in shinbuto did not decrease PGE$_2$ production, but rather, bukuryo increased PGE$_2$ production (Fig. 6A). In similar, kanzo in ninjinto increases annexin1 expression in HGFs but kanzo also inhibits COX activity (Ara and Sogawa, 2016). Residual 2 herbs other than kankyo and kanzo did not decrease PGE$_2$ production (Fig. 6A). Therefore, the increased annexin1 expression could not contribute to decreased PGE$_2$ production.

Both the expression of COX-2 and the productions of IL-6 and IL-8 were widely known to be regulated by NF-κB. Ginger and its components gingerol and shogaol are reported to suppress NF-κB activation, and to decrease COX-2 expression and the productions of IL-6 and IL-8. For example, ginger suppressed NF-κB activation in ovarian cancer cells (Rhode et al., 2007). 6-Gingerol suppressed NF-κB activation in mouse macrophage RAW264.7 cells (Pan et al., 2008), TPA-treated mouse skin in vivo (Kim et al., 2005), and intestinal epithelial cells (Saha et al., 2016). In similar, 6-shogaol suppressed NF-κB activation in mouse macrophage RAW264.7 cells (Pan et al., 2008) and microglia cells (Ha et al., 2012). 6-Gingerol and 6-shogaol suppressed COX-2 expression in mouse macrophage RAW264.7 cells (Pan et al., 2008) and primary rat astrocytes (Shim et al., 2011). 6-Gingerol decreased the productions of IL-1α, IL-1β, IL-6, and IL-8 in intestinal epithelial cells (Saha et al., 2016). However, shinbuto and ninjinto, which contain shokyo and kankyo respectively, increase LPS-induced IL-6 and IL-8 production by HGFs (Fig. 2) similar to hakkonto (Kitamura et al., 2014). Moreover, these two kampo medicines, and shokyo, and kankyo did not suppress COX-2 expression (Figs. 4A and 7). These findings raised the possibility that shokyo and kankyo (or their components, gingerols and shogaols) do not suppress NF-κB pathway in HGFs. The assumption is able to explain that shokyo and kankyo did not suppress COX-2 expression, which is also regulated by NF-κB pathway. In similar, 6-gingerol and 6-shogaol showed no effect on LPS-induced IL-8 production in human bronchial epithelial cells (Podlogar and Verspohl, 2012). Therefore, the effects of gingerols and
shogaols may be different among the kinds of cells.

CONCLUSION

We demonstrated that shinbuto and ninjinto decreased LPS-induced PGE$_2$ production by HGFs. Also shokyo and kankyo, which are included in these kampo medicines respectively, concentration-dependently decreased LPS-induced PGE$_2$ production. However, shokyo and kankyo did not the expression of the molecules in arachidonic acid cascade, suggesting that shokyo and kankyo inhibit cPLA$_2$ activity. Therefore, the kampo medicines that contain shokyo or kankyo may have the ability to decrease PGE$_2$ production. We demonstrate that the kampo medicines used for the patients with deficiency pattern have anti-inflammatory effects as well as those with excess pattern and medium pattern. We expect that kampo medicines are used for the improvement of inflammatory diseases such as periodontal disease and stomatitis in patients with any pattern.

ETHICAL APPROVAL

This study was approved by the Ethical Committee of Matsumoto Dental University (No. 0063).

CONFLICT OF INTERESTS

The authors have no conflicts of interests to disclose.

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**FIGURE LEGENDS**

**Figure 1**
Effects of kampo medicines on the cytotoxicity. HGFs were treated with combinations of LPS (0 and 10 ng/ml) and kampo medicine (0, 0.5, 1, 2, 5, and 10 mg/ml) for 24 h. Then, the numbers of viable cells were measured with WST-8. Open circles, treatment without LPS; closed circles, treatment with 10 ng/ml of LPS. *P < 0.05, **P < 0.01, ***P < 0.001 (without vs. with kampo medicine). P values were calculated by pairwise comparisons and corrected with the Holm method (10 null hypotheses).

**Figure 2**
Effects of kampo medicines on cPLA$_2$, annexin 1, and COX-2 expressions. HGFs were treated with combinations of LPS (0 and 10 ng/ml) and kampo medicine (0, 0.01, 0.1, and 1 mg/ml) for 24 h. Concentrations of PGE$_2$, IL-6, and IL-8 were measured by ELISA, adjusted by cell number, and expressed as per 10,000 cells (mean ± S.D., n = 3). Open circles, treatment without LPS; closed circles, treatment with 10 ng/ml of LPS. *P < 0.05, **P < 0.01, ***P < 0.001 (without vs. with kampo medicine). #P < 0.05, ##P < 0.01, ###P < 0.001 (without LPS vs. with LPS). P values were calculated by pairwise comparisons and corrected with the Holm method (10 null hypotheses).

**Figure 3**
Effects of kampo medicines on COX activity. HGFs were treated with LPS (10 ng/ml) and kampo medicine (1 mg/ml) for 8 h, washed, and then treated with 10 µM arachidonic acid for 30 min. Concentrations of PGE$_2$ were measured by ELISA, adjusted by cell number, and expressed as per 10,000 cells (mean ± S.D., n = 4). P values by Dunnett’s test are indicated.

**Figure 4**
Effects of kampo medicines on cPLA$_2$, annexin 1, and COX-2 expressions. HGFs were treated with a combination of LPS (0 or 10 ng/ml) and kampo medicines (0 or 1 mg/ml) for 8 h, and protein levels were examined by Western blotting. shi, shinbuto; ni, ninjinto.

**Figure 5**
Effects of kampo medicines on LPS-induced ERK phosphorylation. HGFs were untreated (0 h), treated with LPS (10 ng/ml), or treated with both LPS and kampo medicine (1 mg/ml) for 0.5, 1, and 2 h. PMA is used as a positive control. Western blotting was performed using anti-phosphorylated ERK or anti-ERK antibodies. pERK, phosphorylated ERK. Upper band indicates ERK1 (p44 MAPK) and lower band ERK2 (p42 MAPK).

**Figure 6**
Effects of herbs on LPS-induced PGE$_2$ production. (A) HGFs were treated with combinations of LPS (0 and 10 ng/ml) and each herb (100 µg/ml) for 24 h. Concentrations of PGE$_2$ were measured by ELISA, adjusted by cell number, and expressed as per 10,000 cells (mean ± S.D., n = 3). (B) HGFs were treated with combinations of LPS (10 ng/ml) and herb (0, 1, 10, and 100 µg/ml) for 24 h. Concentrations of PGE$_2$ were measured by
ELISA, adjusted by cell number, and expressed as per 10,000 cells (mean ± S.D., n = 3). **P < 0.01, ***P < 0.001 (LPS alone vs. LPS plus herb, Dunnett’s test).

**Figure 7**
Effects of shokyo and kankyo on cPLA₂, annexin 1, and COX-2 expressions. HGFs were treated with a combination of LPS (0 or 10 ng/ml) and herb (1 mg/ml) for 8 h, and protein levels were examined by Western blotting. s, shokyo; k, kankyo.
Table 1 (on next page)

The ingredient of shinbuto formula
Table 1: The ingredient of shinbuto formula

| Japanese name | Latin name                  | Amount (g) | Amount (g/g of product)* |
|---------------|----------------------------|------------|--------------------------|
| bukuryo       | *Poria Sclerotium*         | 4.0        | 0.089                    |
| shyakuyaku    | *Paeoniae Radix*           | 3.0        | 0.067                    |
| sojutsu       | *Atractylodis Lanceae Rhizoma* | 3.0    | 0.067                    |
| shokyo        | *Zingiberis Rhizoma*       | 1.5        | 0.033                    |
| bushi         | *Processi Aconiti Radix*   | 0.5        | 0.011                    |
| total         |                            | 12.0       | 0.267                    |

*7.5 g of shinbuto product contains 2.0 g of a dried extract of the mixed crude drugs.
Table 2 (on next page)

The ingredient of ninjinto formula
Table 2: The ingredient of ninjinto formula

| Japanese name | Latin name                          | Amount (g) | Amount (g/g of product)* |
|---------------|-------------------------------------|------------|--------------------------|
| kankyo        | *Zingiberis Processum Rhizoma*      | 3.0        | 0.083                    |
| kanzo         | *Glycyrrhiza Radix*                 | 3.0        | 0.083                    |
| sojutsu       | *Atractylodis Lanceae Rhizoma*      | 3.0        | 0.083                    |
| ninjin        | *Ginseng Radix*                     | 3.0        | 0.083                    |
| total         |                                     | 12.0       | 0.333                    |

*7.5 g of ninjinto product contains 2.5 g of a dried extract of the mixed crude drugs.
Figure 1 (on next page)

Effects of kampo medicines on the cytotoxicity.
Effects of kampo medicines on cPLA$_2$, annexin 1, and COX-2 expressions.
PGE\(_2\) (pg/10,000 cells)

IL-6 (ng/10,000 cells)

IL-8 (ng/10,000 cells)

kampo (mg/ml)
Figure 3 (on next page)

Effects of kampo medicines on COX activity.
Figure 4 (on next page)

Effects of kampo medicines on cPLA$_2$, annexin 1, and COX-2 expressions.
Figure 5 (on next page)

Effects of kampo medicines on LPS-induced ERK phosphorylation.
| Time (h) | LPS | shinbuto | PMA |
|---------|-----|----------|-----|
| 0       | 0   | 0.5      | 0.5 |
| 0.5     | 1   | 1        | 1   |
| 1       | 2   | 2        | 2   |
| 2       | 0.5 | 0.5 (h)  |      |

**Western Blot Analysis**

- **pERK**
- **ERK**
Figure 6 (on next page)

Effects of herbs on LPS-induced PGE$_2$ production.
Figure 7 (on next page)

Effects of shokyo and kankyo on cPLA$_2$, annexin 1, and COX-2 expressions.
