In Vitro and in Vivo Studies on Anti-Inflammatory Effects of Traditional Okinawan Vegetable Methanol Extracts

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ABSTRACT: To determine anti-inflammatory effects of traditional Okinawan vegetable methanol extracts, we examined the inflammatory mediators from mouse macrophage by the addition of extracts prepared from two kinds of nishi-yomogi and hosoba-wadan. Moreover, we inspected the impact of extracts using a carrageenin-induced acute inflammatory animal model. In the case of in vitro study, nitric oxide and cytokine concentrations of supernatant were analyzed with Griess reagent and a commercially available enzyme-linked immunosorbent assay kit, respectively. Gene expressions related to the inflammation were extrapolated from the data of real-time polymerase chain reaction. In the case of in vivo study, six-week-old male Sprague Dawley rats were intraperitoneally administered with vegetable extracts, and then developed edema by carrageenin administration on the footpad. The footpad volume was obtained from the volume in before and after carrageenin administration. In the case of in vitro study, nitric oxide and tumor necrosis factor-α concentrations of the Kume nishi-yomogi were significantly lower (P < 0.05) than those of other groups, and also interleukin-6 concentration of the Kume nishi-yomogi was significantly lower (P < 0.05) than that of control. The iNOS and COX-2 gene expressions of Kume nishi-yomogi were significantly lower (P < 0.05) than those of other groups. In the case of in vivo study, the footpad volume between groups showed no significant difference; however, the decreased tendency of the footpad volume in Kume nishi-yomogi was observed as compared with other groups. From these results, it is expected that Kume nishi-yomogi extract might have a physiological function in the alleviating inflammation.

1. INTRODUCTION

Okinawa is well known as a longevity prefecture in Japan. Therefore, it is of great interest to investigate the relationship between health or longevity and the consumption of distinctive Okinawan foods. In particular, there are many regionally specific agricultural crops in Okinawa. Among these, 28 agricultural crops are known as “traditional Okinawan agricultural crops (shima yasai)”, which are defined as follows: (i) they have been used since before World War II, (ii) they are commonly used in local cuisine, and (iii) they are suitable for the climate of Okinawa. These crops contain relatively large amounts of polyphenols comprising several subtypes.

Recently, it has been suggested that the polyphenols derived from natural products have various physiological functions, such as antioxidant, anti-inflammatory, antiallergic, and anticarcinogenic. However, there have been few information on the physiological effects of polyphenols found in traditional Okinawan vegetables. Therefore, we are currently conducting research focusing on polyphenols contained in traditional Okinawan vegetables for anti-inflammatory, anti-allergic, anticancer, and immunostimulatory activities.

Particularly, among physiological functions of polyphenols, anti-inflammation is thought to be related to the antioxidant capacity of polyphenols, as their main mechanisms involved in the inflammatory reaction are closely associated with the derived free radicals and the oxidative stress. Therefore, it is interesting if the polyphenols contained in traditional Okinawan vegetables are involved in anti-inflammatory responses.

Inflammation is one of the biological responses of body tissues to harmful stimuli and is a protective response for humans. It can be classified either acute or chronic inflammation. As Hwang et al. describe in their paper, the inflammation is accompanied by the development of various mediators such as nitric oxide (NO) and proinflammatory cytokines, which are associated with the typical symptoms of inflammation such as pain, heat, redness, swelling, and loss of function. NO is generated by the action of inducible NO synthase (iNOS), while the proinflammatory cytokine productions such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α are regulated by nuclear factor-kB (NF-kB), which is a key mediator of inflammation. The NF-kB translocates to the nucleus, where it binds with the kB-binding site in the promoter regions of target gene and induces the transcription of proinflammatory cytokines.

Thus, the inhibition of the NF-kB pathway is thought to be a mechanism that contributes to the improvement of typical symptoms of acute or chronic inflammation.
In this paper, therefore, we introduced the anti-inflammatory effect of the traditional Okinawan vegetables with distinct polyphenol profiles such as Crepidiastrum Lanceolatum Nakai (hosoba-wadan) and two kinds of Artemisia indica Willd. var. orientalis (Pamp.) Hara hara (nishi-yomogi) sampled from Kume and Ishigaki islands. We examined the effects of traditional Okinawan vegetable extracts on the proinflammatory mediator productions by mouse macrophage RAW 246 cells and the gene expressions of NF-κB, COX-2, and iNOS that are associated with inflammation and NO production, respectively. In addition to the in vitro study, the carrageenin-induced footpad edema animal model was used to investigate the effect of intraperitoneal administration of traditional Okinawan vegetable methanol extracts on edema.

2. RESULTS AND DISCUSSION

2.1. Effects of Traditional Okinawan Vegetable Methanol Extracts on Cell Viability. RAW264 cell viability after addition of methanol extracts is shown in Figure 1. Cell viability or cytotoxicity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability was reduced in a concentration-dependent manner with all vegetable extracts. All methanol extracts of Okinawan vegetables did not show significant cytotoxicity at concentrations of 1 μg/mL as compared with that of control. Based on these results, all extracts were prepared at a concentration of 1 μg/mL ethanol and were added to assess their influence on anti-inflammation.

2.2. Polyphenol Profiles of Hosoba-wadan, Kume, and Ishigaki Nishi-yomogi Methanol Extracts by HPLC Analysis. The polyphenol profiles of hosoba-wadan and nishi-yomogi methanol extracts were analyzed by HPLC (Figure 2). The primary components of the hosoba-wadan methanol extract were chlorogenic acid, chicoric acid, and luteolin glycosides, such as luteolin 7-O-β-D-glucopyranoside and luteolin 7-O-β-D-gluconuronide, while those of the nishi-yomogi methanol extract were mainly composed of chlorogenic acid, caffeoylquinic acid, and iso-chlorogenic acids, such as 3,5-, 4,5-, and 3,4-dicaffeoylquinic acids. The characteristic difference between the polyphenol profiles of Ishigaki and Kume nishi-yomogi was only the presence or not of an unknown peak detected at a retention time of 22.45 min.

2.3. Concentrations of NO and Cytokines (IL-6 and TNF-α) Released from RAW264 Cells. Figure 3 shows the concentrations of NO and preinflammatory mediators, such as IL-6 and TNF-α, released from RAW264 cells into medium. All mediator concentrations of nishi-yomogi collected in Kume island were significantly lower (P < 0.05) than those of other groups. These markers are well known as inflammatory mediators and proinflammatory cytokines. Therefore, it is thought that the suppression of these marker productions from RAW264 cells is associated with reduction or suppression of inflammation. These results suggest that the Kume nishi-yomogi methanol extract may have anti-inflammatory effect though the main components involved in the effect could not be clearly identified.

2.4. Gene Expressions Related to Inflammation such as iNOS, COX-2, and NF-κB. The gene expressions related to inflammation are illustrated in Figure 4. The mRNA expression levels of iNOS and COX-2 in the Kume nishi-yomogi group were significantly lower (P < 0.05) than those in the other groups. However, there was no significant difference in the mRNA level of NF-κB among groups. In addition to the effects on preinflammatory and inflammatory marker productions of Okinawan vegetable methanol extracts, the influence of the Kume nishi-yomogi extract was also observed on the gene expressions associated with inflammation. NO is generated by the enzymatic action of inducible NO synthase (iNOS) during inflammation, and COX-2 is an enzyme induced at the site of inflammation. From these results, it seems that the suppression of inflammation marker productions by the addition of the Kume nishi-yomogi extract to RAW264 cells is associated with the downregulation of gene expressions related to inflammation. However, we could not detect significant influence on the gene expression of NF-κB, which is a nuclear transfer factor that plays a pivotal role in preinflammatory cytokine production.

2.5. Carrageenin-Induced Rat Footpad Edema. As shown in Figure 5, although no significant difference was detected in footpad edema of rats treated with intravenous administration of vegetable extracts among groups, intraperitoneal administration of the Kume nishi-yomogi extract was the most effective in alleviating rat footpad edema among all groups. The alleviative effect of edema by hosoba-wadan was less effective than that by the Kume nishi-yomogi extract. The carrageenin-induced footpad edema is an experimental animal model to evaluate the anti-inflammatory medication and often used to evaluate the alleviation effect of edema on acute inflammation. Unfortunately, in this study, we could not observe significant alleviation of edema by administration of vegetable extracts in animal models. However, the dose of extract, timing of administration, and some other problems have not been sufficiently studied, so these are needed to be improved in the future.

3. EXPERIMENTAL MATERIALS AND METHODS

3.1. Polyphenol Preparation from Traditional Okinawan Vegetables. Freeze-dried hosoba-wadan and nishi-yomogi cultivated at Okinawa Prefecture Agricultural Research Center, Japan was extracted twice (50 °C, 1 h) with methanol, and the solvent was evaporated. The polyphenol contents of Okinawan vegetable methanol extracts were approximately 5 mg/g for hosoba-wadan and 20–30 mg/g for nishi-yomogi. To
adjust a concentration of 1 mg/mL for the in vitro study, extracts were resolved with an appropriate volume of special grade ethanol (99.5%, Wako Pure Chemical Industries, Ltd. Osaka, Japan).

3.2. Determination of Total Polyphenol and Polyphenol Profiles by HPLC Analysis. The total polyphenol content of vegetable methanol extracts was determined by the Folin–Denis method with minor modifications (Folin et al., 1912). Briefly, a Folin & Ciocalteu’s phenol reagent (20 μL) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), an aqueous solution of 10% Na2CO3 (40 μL) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and distilled water (120 μL) were added to 20 μL of the methanol extract and the mixture was incubated at room temperature for 1 h under dark conditions. The absorbance was measured at 750 nm with a spectrophotometer. An aqueous solution of gallic acid was used as a standard.

Polyphenols in the hosoba-wadan and nishi-yomogi methanol extracts were determined by HPLC using a Mightysil RP-18 GP II (5 μm) column (4.6 × 250 mm²; Kanto Chemical Co., Inc., Japan) at a flow rate of 1.0 mL/min. The sample was injected, and elution was performed with a system composed of solvent A (0.1% phosphoric acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in water) and solvent B (0.1% phosphoric acid in acetonitrile/methanol solution mixed at the same volume). The elution was detected by a UV–vis detector at 280 nm. Compounds corresponding to each peak were

Figure 2. Polyphenol profiles of traditional Okinawan methanol extracts by HPLC analysis. The HPLC charts show (A) hosoba-wadan, (B) Kume nishi-yomogi, and (C) Ishigaki nishi-yomogi.

Figure 3. NO and cytokine (IL-6 and TNF-α) concentrations in the medium produced by the addition of traditional Okinawan vegetable methanol extracts at a final concentration of 1 μg/mL. (A) NO, (B) IL-6, and (C) TNF-α concentrations. Data are shown as means ± SEM for three treatments. Comparison between groups was conducted by the Tukey–Kramer test, and different letters were used to indicate statistically significant differences between groups at P < 0.05.
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**Figure 4.** Effects of the traditional Okinawan vegetable methanol extracts on the gene expressions related to inflammation such as NF-κB (A), iNOS (B), and COX-2 (C). RAW264 cells were treated with the extracts of a final concentration of 1 μg/mL. Data are shown as means ± SEM for three treatments. Comparison between groups was conducted by the Tukey–Kramer test, and different letters were used to indicate statistically significant differences between groups at P < 0.05.

**Figure 5.** Effects of traditional Okinawan vegetable methanol extracts on carrageenin-induced rat footpad edema. Prior to induction of footpad edema, rats were given 200 μL of 1 mg/mL extracts intraperitoneally.

identified by comparison with the retention time of reference standards and our previous report.12

### 3.3. Cell Culture.

RAW264 cells, obtained from the RIKEN Cell Bank (RCB0535, Tsukuba, Japan), were cultured in Dulbecco’s modified Eagle medium (high glucose) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% (v/v) fetal bovine serum (Sigma-Aldrich Co., St. Louis, MO), penicillin (100 U/mL) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and streptomycin (100 μL/mL) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in a humidified atmosphere with 5% CO2 at 37°C. Some cells were subcultured for the MTT assay after trypsinization (0.25% trypsin-ethylenediaminetetraacetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution was added to each well to solubilize formazan crystals, and absorbance was then measured at 595 nm (iMark, BioRad, Hercules, CA). The density of formazan formed in the control group was calculated as 100% viability.

### 3.5. Measurement of NO and Cytokine (IL-6 and TNF-α) Concentrations in the Medium.

RAW264 cells were treated with 1 μg/mL of Okinawan vegetable methanol extracts for 24 h. After incubation, the supernatant was collected and used for NO and cytokine assays. In the case of the NO assay, the supernatants were mixed with Griess reagent containing equal volumes of 1% sulfanilamide (Wako Pure Chemical Industry, Ltd., Osaka, Japan) in 2.5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride (Wako Pure Chemical Industry, Ltd., Osaka, Japan) solution and then incubated at room temperature for 10 min. The concentration of nitrite oxide was measured by OD at 595 nm. NaN3 (Wako Pure Chemical Industry, Ltd., Osaka, Japan) was used as a standard reagent, while the determination of cytokine concentrations was measured using a Quantikine enzyme-linked immunosorbent assay kit (R&D Systems, Inc., Minneapolis, MN) for IL-6 and TNF-α following the manufacturer’s instructions.

### 3.6. Real-Time Polymerase Chain Reaction (PCR).

In this study, we examined the gene expression related to inflammation such as iNOS, COX-2, and NF-κB. Total RNA was extracted from RAW264 cells using Trizol reagent (Promega, Madison, WI). Then, the cDNAs were synthesized with high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Reactions were performed with Power SYBR Green PCR Master Mix and a Step One real-time PCR system (Applied Biosystems, Foster City, CA). Results were normalized by the number of target DNA copies to that of β-actin gene by simplification of the comparative threshold cycle (ΔΔCt) method. Rat-specific primer pairs are listed Table 1.

### 3.7. Carrageenin-Induced Rat Footpad Edema.

Male Sprague Dawley rats weighing 160–180 g were purchased from Japan SLC (Shizuoka, Japan). They were individually housed at 23 ± 1°C on a 12 h light cycle and with free access...
Table 1. Primer List of Real-Time PCR

| Primer      | Sequence                  |
|-------------|---------------------------|
| β-act forward | 5'-CTCTGGGCTCCTACGAGACTGAGAGA-3' |
| reverse      | 5'-GAAACACGCTCAGTTAAGCACT-3'   |
| NF-κB forward | 5'-AGCCAGCTCCGTGTTGTTGTT-3'    |
| reverse      | 5'-AGGGTTCCGTTCCACTGATTTCC-3'  |
| iNOS forward | 5'-TAGGACGAGATTTGGAGGCCCTG-3'  |
| reverse      | 5'-GGGTGTTGTGCGAACCTCCAGTC-3'  |
| COX-2 forward | 5'-CAGGTCATTGGTGGAGAGGTTG-3'   |
| reverse      | 5'-TGCTCATCACCCCCCACCTCAGG-3'  |

to commercial chow (CE-2, Crea Japan Inc., Japan) and tap water. This experiment was conducted in compliance with the ethical guidelines of the Fukuoka Institute of Technology Animal Care and Use Committee (approval number: 20180925-0001).

Prior to carrageenin administration, rats were treated with 200 μL of 1 mg/mL extracts by intraperitoneal administration. Two hours later, footpad edema was induced in the hind right paw of rat by an intraplantar injection of 100 μL of freshly prepared carrageenin (1% solution in sterile saline). Edema was measured with the use of a plethysmometer (model MK-101 P, Muromachi Kikai Co., Ltd., Japan) at 1 h intervals up to 4 h after carrageenin injection. The results are expressed by volume as the difference of swelling before and after carrageenin administration.

3.8. Statistical Analysis. Data are shown as means ± SEM (standard errors of the mean). In the case of the MTT assay, data were assessed by one-way analysis of variance and then references were done for comparisons between groups. Differences were considered to be significant at P < 0.05.

4. CONCLUSIONS

The results of in vitro study are summarized in Figure 6. In the case of in vitro study, we observed that the addition of the vegetable extracts on the reduction of footpad edema in the carrageenin-induced animal model.

In addition to these studies, we are currently considering the effect of toll-like receptor (TLR)-4 and NF-κB expressions using Western blot analysis. Moreover, in the future, we plan to verify the active components and examine the effects of daily intake of traditional Okinawan vegetables on chronic inflammation.

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Notes
The authors declare no competing financial interest.

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