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Ingestion of *Tabebuia avellanedae* (Taheebo) Inhibits Production of Reactive Oxygen Species from Human Peripheral Blood Neutrophils

Research Article

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Abstract

Neutrophils are a main source of oxidative stress by reactive oxygen species (ROS), and the tissue damage caused of excessive ROS might lead to aging and many diseases. Whereas the anti-inflammatory effects of *Tabebuia avellanedae* (taheebo) have been reported *in vitro* and in animal experiments, little is known in humans. Fourteen adult volunteers took 4 capsules (total 500 mg) of taheebo extracts (Taheebo Japan, Co., Ltd., Osaka, Japan) per day orally for 2 weeks. The production of ROS from peripheral blood neutrophils was measured by luminol-dependent chemiluminescence (LmCL) in a kinetic mode at 30-minute intervals for 1.5 hours with a luminometer at 37˚C. As a result, 1.5-hour point, peak and sum of values of LmCL were significantly decreased at 2 weeks after administration of taheebo extracts. In conclusion, we demonstrated that administration of *Tabebuia avellanedae* inhibited ROS production from human neutrophils.

Keywords: *Tabebuia avellanedae*; Oxidative Stress; Neutrophil; Luminol-Dependent Chemiluminescence; Inflammation; Mebiol Gel.

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Introduction

Reactive oxygen species (ROS) are known to play a dual role in biological systems since they can be either harmful or beneficial to living systems [1]. Beneficial effects of ROS involve physiological roles in cellular responses in host defense against infectious agents and in the function of a number of cellular signaling systems. In contrast, excessive ROS can be important mediators of damage to cell structures, including lipids, proteins and nucleic acids. Furthermore, the damages might lead to aging and many diseases such as chronic inflammatory disease, cardiovascular disease and cancer [2, 3].

Neutrophils are a main source of ROS production, especially in inflammatory reactions. Through phagocytosis, neutrophils play an important role in host defense against invading pathogens and are the major effectors of the acute inflammatory reactions. In response to various agents, neutrophils release large quantities of superoxide anion (O_2^-) in a phenomenon known as respiratory burst [1]. The inappropriate activation of respiratory burst is associated with tissue injury and impairment of the ability to clear invading microorganisms. Thus, the balance between beneficial and harmful effects of neutrophil functions should be properly modulated.

In order to prevent various diseases caused by excess ROS, functional natural products, which suppress production of ROS or scavenge ROS, have been receiving much attention. One of the functional natural products is *Tabebuia avellanedae*, which is a broadleaf tree of the department of a trumpet creeper and grows wild in South America Brazil and an Amazonian watershed. The water extracts of the inner bark of *Tabebuia avellanedae* was called "taheebo" generally, and taheebo has been drunk as tea traditionally. *Tabebuia avellanedae* has also been used for various ethopharmacological treatments of bacterial infection, blood coagulation and cancer [4, 5]. Moreover, recent studies have shown that *Tabebuia avellanedae* has some effects of anti-inflammation or anti-oxidation [6-8].

Although the anti-inflammatory effects of *Tabebuia avellanedae* have been reported *in vitro* and in animals, little is known in humans. In addition, there is no report that *Tabebuia avellanedae* inhibits neutrophil activation. To address these issues, the purpose of this study was to investigate that *Tabebuia avellanedae* extract modulated neutrophil function in humans by assessment of luminol-dependent chemiluminescence (LmCL), which largely detects

myeloperoxidase (MPO)-dependent formation of highly toxic ROS such as hypochlorous acid (HOCI) [9, 10].

Materials and Methods

Subjects

The entry criteria were as follows: healthy adults; no use of dietary supplements; no severe organ function impairment and no chronic diseases. Prior to enrollment, all subjects were provided and required to sign written informed consent. The protocol was approved by the Ethical Committee at Waseda University.

Study protocols

The chosen test product, "TAHEEBO NAFDIN® soft capsule (Taheebo Japan, Co., Ltd., Osaka, Japan)", contains 125 mg *Tabebuia avellanedae* extract per capsule. One capsule contains the following: carbohydrates 127.5mg, protein 115mg, fat 195mg, sodium 0.41mg, water 12.5mg, Ca 0.71mg, Fe 0.04mg, K 0.93mg, P 0.20mg, and Mg 0.28mg. Results from tests for heavy metals (mercury, cadmium, lead and arsenic) conformed to strict Japanese food regulations. We did an open-label study in which 4 capsules of TAHEEBO NAFDIN® were given per day orally for 2 weeks. To establish an accurate baseline level of ROS production by peripheral blood neutrophils before the initiation of treatment, peripheral blood samples were collected before administration. After administration of TAHEEBO NAFDIN®, peripheral blood samples were obtained on the 1st, 7th and 14th days.

Synthesis of peptide-bound temperature-responsive polymer (G-TRP)

Twenty-four grams of collagen peptide (SCP-5100; Nitta Gelatin Co., Osaka, Japan) were dissolved in 96 g of distilled water at 37˚C, followed by reaction with 3.26 g of N-acryloylsuccinimide (Kokusan Kagaku Co., Ltd., Tokyo, Japan) for 4 days at 37˚C to obtain polymerizable collagen peptide. N-Isopropylacrylamide (108.5 g; Kojin Co., Tokyo, Japan) and n-butyl methacrylate (4.26 g; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were dissolved in 600ml of ethanol and then 123g of the above aqueous solution of polymerizable collagen peptide was added. Under nitrogen atmosphere, 1ml of N, N, N', N'- tetramethylethylenediamine was added to the mixed solution (Wako Chemical) and 10 ml of 10 wt% ammonium persulfate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) aqueous solution reacted for 5 hours at 4˚C, maintaining the nitrogen atmosphere. After the reaction, 30 L of cold (4˚C) distilled water were added and the mixture concentrated to 3 L using an ultrafiltration membrane (molecular weight cut off of 100,000) at 4˚C. This dilution and concentration process was repeated 5 times in order to remove impurities and low molecular species. Lyophilization and sterilization of the final concentrated solution gave 105g of peptide-bound temperature-responsive polymer (G-TRP).

Preparation of scaffold-thermoreversible gelation polymer (S-TGP) gel

Under a clean-air laminar hood workbench, 0.5g of G-TRP and 0.5g of thermoreversible gelation polymer (Mebiol Gel; Mebiol Inc, Kanagawa, Japan) was dissolved in 16.7 ml of Hank's Balanced Salt Solution (HBSS, calcium chloride, magnesium chloride) at 4˚C overnight, yielding a viscous transparent scaffold-thermoreversible gelation polymer (S-TGP) gel of uniform liquid without any bubbles for use in the experiments [11]. Mebiol Gel is a pure synthesized biocompatible copolymer composed of thermoresponsive polymer blocks and hydrophilic polymer blocks, characterized by its temperature-dependent dynamic viscoelastic properties and used as a biocompatible scaffold for three-dimensional culture without any toxicity. S-TGP gel is a peptide-bound thermoreversible gel formed by mixing Mebiol Gel and G-TRP.

Luminol-dependent chemiluminescence (LmCL) assay

Peripheral blood samples were obtained from subjects using Na-heparin containing tubes (Terumo Venoject II, Terumo Co, Tokyo, Japan). An aqueous solution of S-TGP gel was solidified by raising its temperature. Accordingly, 50 µl S-TGP gel was dispensed into micro tubes (2 ml), and spread carefully at 4˚C, and set on block incubators at 37˚C. In addition, the blood samples were mixed with 2.5 mM luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Aldrich, MO, USA) at a ratio of 1:1. The luminol-blood samples (150µl) were set on the S-TGP gel tubes at 37˚C. The production of ROS from neutrophils were detected as the values of LmCL using a luminometer (Gene Light 55; Microtec Co., Ltd., Funabashi, Japan), in a kinetic mode at 0, 0.5, 1.0 and 1.5 hours. Neutrophils migrate from the blood into the S-TGP gel in the tube at 37°C, and LmCL can be detected through the transmissive gel, thereby there is no need to separate neutrophils from blood to determine ROS production reducing any delay in sample processing that is associated with conventional methods [9]. After LmCL was measured at 1.5 hours, luminol-blood samples in the tubes were removed and the tubes with 50 µl S-TGP in which neutrophils migrated were washed three times with PBS warmed at 37°C. Then, the tubes with gel were cooled on ice, and 50 µl reagent A and 50 µl reagent B (ChemoMetec A/S, Allerød, Denmark) were added and mixed well, which effectively makes the cell membrane permeable to the DNA staining dye, and is effective in the dispersion of cell aggregates. The samples were aspirated into a NucleoCassett and the cell number was counted by the NucleoCounter (ChemoMetec A/S, Allerød, Denmark).

Statistical analysis

The Shapiro-Wilk test was used to check for normality of distribution. Statistical validation was carried out with the Friedman repeated measures analysis of variance on ranks and with post hoc multiple pairwise comparison for Friedman. Calculations were performed using IBM SPSS Statistics version 19.

Results

Subjects' characteristics

The total number of subjects enrolled for assessment was 14. The 14 volunteers had a mean age of 43 with ages ranging from 30 to 57. The gender distribution of male and female was 2 and 12, respectively.

The LmCL values at each point

The LmCL values at 0.5 and 1.0 hour points were not significantly changed by 2-week intake of TAHEEBO NAFDIN® (Figures 1,

Figure 1-7. Box and whisker plots demonstrating luminol-dependent chemiluminescence (LmCL) and cell counts samples from volunteers for 2-week intake of taheebo: changes for 2 weeks of LmCL at 0.5 hours (1), 1.0 hours (2), 1.5 hours (3), peak value during measurement (4), sum of values at 1.5 hours (5), count of migratory cells into gel at 1.5 hours (6) and sum of values per a cell at 1.5 hours (7). P-values were calculated by post hoc multiple pairwise comparison for Friedman. Box contains values between 25th and 75th percentiles of LmCL (central line, median). Vertical lines represent the 10th and 90th percentiles.

Figure 1

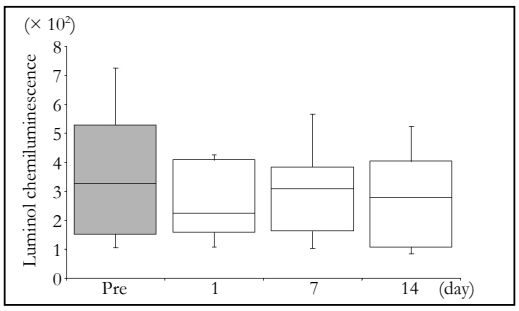


Figure 2

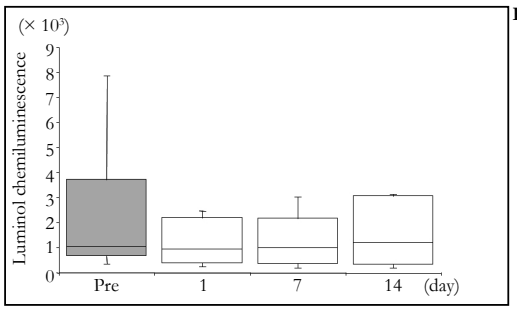


Figure 3

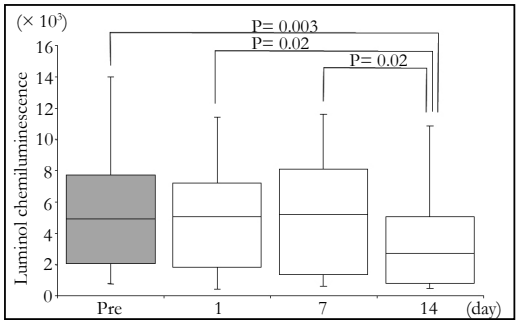


Figure 4

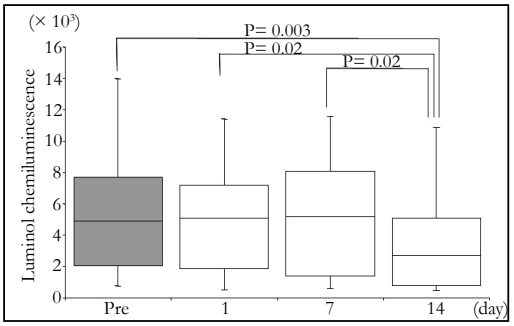


Figure 5

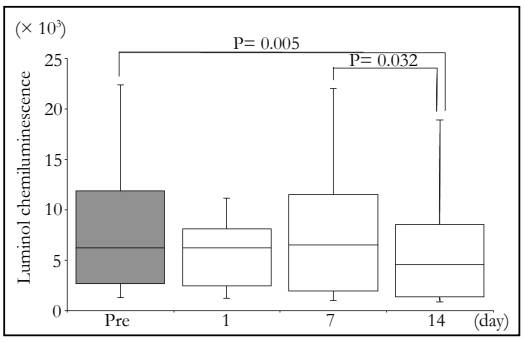


Figure 6

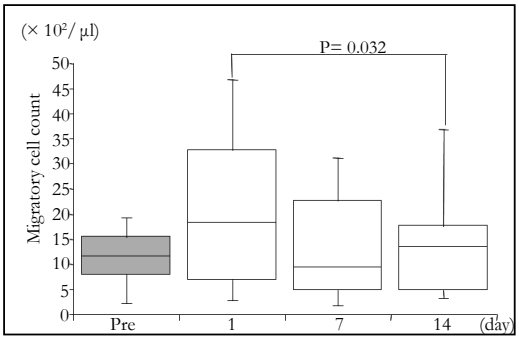
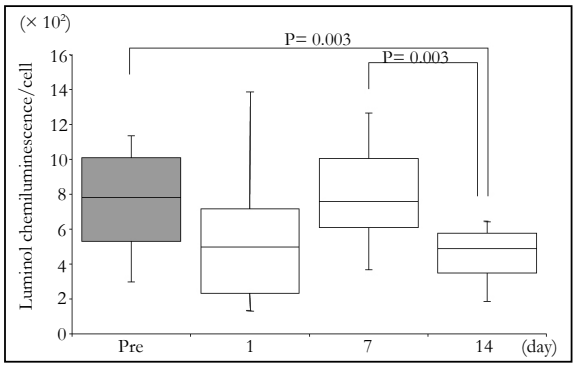


Figure 7



2). The LmCL value at 1.5-hour point was markedly decreased by 2-week intake of TAHEEBO NAFDIN® (Friedman: p = 0.002) (Figure 3).

Peak value in LmCL

The peak value in LmCL was assessed during measurement (0.5–1.5 hours). The peak value in LmCL was markedly decreased in volunteers by 2-week intake of TAHEEBO NAFDIN® (Friedman: p = 0.004) (Figure 4).

Sum of value in LmCL

The total LmCL was determined from the sum of LmCL values at 0.5-1.5 hours. Total LmCL at 1.5 hours was significantly decreased by 2-week intake of TAHEEBO NAFDIN® (Friedman: p = 0.002) (Figure 5).

Number of migratory neutrophils

The number of migrated cells was not significantly influenced by 2-week administration of TAHEEBO NAFDIN® (Figure 6).

When adjusted by migrated cell count, the LmCL values per cell basis were still inhibited by TAHEEBO NAFDIN® intake for 2 weeks (Friedman: $p < 0.001$) (Figure 7).

Discussion

This study demonstrated that the LmCL values were significantly decreased in volunteers after 2-week intake compared with pre administration of *Tabebuia avellanedae* extract. Even if it was adjusted for this influence with the number of migratory cells, it was still significant. These results suggest that *Tabebuia avellanedae* did not inhibit neutrophil migration, but the ROS produced by migrated cells was significantly suppressed.

The neutrophil activation may be controlled by *Tabebuia avellanedae* extracts based on the anti-inflammatory or the antioxidant effect. In the past, a few reports have been available on the anti-inflammatory effect of *Tabebuia avellanedae*. Byeon et al. demonstrated that *Tabebuia avellanedae* negatively modulated macrophage-mediated inflammatory responses by suppressing PGE_2 production [6]. Awale et al. reported that *Tabebuia avellanedae* suppressed the activity of macrophage-like J774.1 cells [8]. Neutrophils play an important role like a macrophage in inflammation. However, most studies did not focused on neutrophil functions controlled by *Tabebuia avellanedae*. We demonstrated that *Tabebuia avellanedae* inhibits production of ROS in human neutrophils.

The major active compounds of *Tabebuia avellanedae* are furanonaphthoquinones, quinones (lapachol and β-lapachone), naphthoquinones, flavonoids, iridoids and phenolic glycosides. From the water extract of *Tabebuia avellanedae*, iridoids and phenylethanoid glycoside have showed the inhibitory activities of nitric oxide (NO) production in LPS-activated macrophage-like J774.1 cells [8]. Furano naphthoquinones and β-lapachone may mediate immune modulating activities *in vitro* [12]. Although these compounds of *Tabebuia avellanedae* might be the candidate, additional research is needed to determine which controls activation of the neutrophils.

In this study, we demonstrated that *Tabebuia avellanedae* suppressed activation of neutrophils in humans by LmCL assessment. Neutrophil respiratory burst activity can be measured in the presence of a light emitting reporter molecule by the production of NADPH oxidase-dependent oxidants such as O_2^-, hydrogen peroxide (H_2O_2) and MPO-dependent hypochlorous acid (HOCl) production using luminol as the lumiphor [13]. Although we used Mebiol gel as thermoreversible gelation polymer, it is useful because of the characteristics, which liquefy at low temperature, turn to gel immediately upon warming and return to a liquid state again when cooled. The greatest benefit of our method is its rapidity and simplicity of analysis using an analytical protocol, which does not require any cell separation procedure. In addition, our assay system using the cellular response of neutrophils to assess the state of neutrophil activation within the milieu of the immunologic profile may be applicable for screening antioxidant materials for estimating anti-inflammatory and/or antioxidant activity. These insights are fostering new anti-inflammatory therapeutic approaches to inflammation-related disease.

Conclusion

We demonstrated that *Tabebuia avellanedae* extract inhibited ROS production of neutrophils in humans. For the future, it is necessary to investigate the mechanisms and active substance of *Tabebuia avellanedae* relevant to modulation of neutrophil function.

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