FP-FST-006-ID016

Immature Black Vinegar Extract Activates Macrophages

Eri Ishii[1], Kosuke Nishi[1,2], Momoko Ishida[1], Masanobu Nagano[3], Kazunori Hashiguchi[3], Akira Fujii[3] and Takuya Sugahara[1,2,*]

1 Graduate School of Agriculture, Ehime University, Matsuyama, Ehime 790-8566, Japan
2 Food and Health Sciences Research Center, Ehime University, Matsuyama, Ehime 790-8566, Japan
3 Sakamoto Kurozu Inc., 21-15 Uenosono-cho, Kagoshima 890-0052, Japan

* Corresponding author: mars95@agr.ehime-u.ac.jp

Received: 9 July 2018; Accepted: 4 December 2018; Published: 6 January 2020

Abstract: Black vinegar is a traditional rice vinegar produced in Kagoshima prefecture in Japan. This vinegar is produced by saccharification, fermentation, and aging in one pot. Wakazu is an immature black vinegar collected before aging for 1-3 years. Many researchers are studying on health functions of black vinegar, and various functions have been revealed such as antitumor and antiallergic effects. However, the studies on wakazu have not been done yet. Therefore, in this study, the immunostimulatory effect of wakazu on macrophages was investigated. Acetic acid was removed from wakazu by repeated freeze-drying. After centrifugation to remove insoluble substances, wakazu was dialyzed with a 14 kDa molecular weight cut off dialysis membrane against 10 mM sodium phosphate buffer before use. As a result, wakazu enhanced TNF-α and IL-6 production by mouse macrophage cell line, RAW264.7 cells. In addition, wakazu stimulated gene expression of these cytokines in RAW264.7 cells. Western blot analysis indicated that wakazu enhances translocation of NF-κB to nucleus and MAP kinase signal transduction in RAW264.7 cells. In conclusion, immature black vinegar, wakazu also has a potential as a health-promoting food with the immunostimulatory effect.

Keywords: Black vinegar; Macrophage; Cytokine production; IL-6; TNF-α

1. Introduction

Macrophages are versatile cells that play crucial roles in the innate immune system for host defense. Macrophage activation is mediated primarily by recognition of active substances through specific receptors in the initial phase of the immune response. Macrophages bind to the activator via Toll-like receptor (TLR) 4, CD14, complement receptor 3, scavenger receptor, dectin-1, or mannose receptor [1]. This process initiates the activation of intracellular signaling cascades including mitogen-activated protein (MAP) kinases and NF-κB, thereby inducing macrophage activation. The activated macrophages secrete several mediators including inflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α and release some cytotoxic and inflammatory molecules such as nitric oxide (NO) [2]. These cytokines stimulate the antibody production by plasma cells and the synthesis of other inflammatory mediators that have antitumor and antibacterial activities. Furthermore, macrophages have phagocytosis activity which induces the activation of T cells and B cells and contribute to the activation of the adaptive immune response. Therefore, the activation of macrophages is very important in enhancing the entire immune system both of the innate and adaptive immune responses.

The black vinegar is a traditional vinegar manufactured with three components: steamed unpolished rice, a fermentation starter called koji, and water. Various microorganisms such as Aspergillus, yeasts, lactic acid bacteria, and acetic acid bacteria are involved in their process of fermentation and maturation such as saccharification, alcoholic fermentation, and acetic fermentation.

Wakazu is an immature black vinegar collected before aging for 1-3 years, and characterized by a light amber color and sour than black vinegar. Many researchers are studying on health functions of black vinegar, and various functions have been revealed such as antioxidative activity [3] and antiallergy effect [4]. However, the studies on wakazu have not been done yet. Therefore, in this study, the immunostimulatory effect of wakazu on macrophages was investigated.

2. Materials and methods

2.1. Sample preparation

Wakazu manufactured by Sakamoto Kurozu Inc. (Kagoshima, Japan) was repeatedly freeze-dried to remove acetic acid. It was then centrifuged at 1,500 × g for 10 min to remove insoluble substances, and the supernatant was collected and dialyzed with a 14,000 molecular weight cut off dialysis membrane (Wako Pure Chemical Industries, Osaka, Japan) against 10 mM sodium phosphate buffer (NaPB) for 24 h at 10°C. The dialyzed supernatant was filtrated through a 0.22 μm membrane, and it was passed through Endo Trap (Hyglos GmbH - a bioMérieux company, Freistaat Bayern, Germany), which is an affinity matric intended for removal of lipopolysaccharide from biological samples in aqueous solutions. Dry weight of wakazu components was measured after freeze-drying of wakazu preparation.

2.2 Cells and cell culture

RAW 264.7 cells, a mouse macrophage-like cell line, were obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). RAW264.7 cells were cultured in DMEM supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) at 37°C under humidified 5% CO2. RAW264.7 cells were detached using phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.02% ethylenediamine-N,N,N',N'-tetraacetic acid (Dojindo Laboratories, Kumamoto, Japan) for the subsequent experiments.

2.3. Determination of cytokine levels in culture medium

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 96-well cell culture plate (Corning, Corning, NY, USA) at 6.0 × 10^4 cells/well and cultured for 16 h at 37°C. After washing with PBS, the cells were treated with 200 μL of 10% FBS-DMEM containing various concentrations of wakazu, 10 mM NaPB as negative control, or 100 ng/mL of LPS from ***E. coli*** O26/B6 (Sigma-Aldrich) as positive control and incubated for 6 h at 37°C. After incubation, the concentrations of IL-6 and TNF-α in the culture media were measured by **m**ouse IL-6 ELISA **kit** (Bio Legend, San Diego, CA, USA) and Mouse TNFα ELISA **kit** (eBioscience, San Diego, CA, USA), respectively.

2.4. Cell viability

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 96-well cell culture plate at 6.0 × 10^4 cells/well and **cultured for 16 h at 37°C**. After washing with PBS, the cells were treated with 200 μL of 10% FBS-DMEM containing various concentrations of wakazu, 10 mM NaPB as negative control, or 100 ng/mL of LPS as positive control and **incubated for 6 h at 37°C**. After incubation, the cell supernatant was collected for ELISA, and the cells were washed with PBS twice. Cell viability was measured using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan).

2.5. Real time RT-PCR

RAW264.7 cells suspended **in** 10% FBS-DMEM were seeded into a 24-well cell culture plate (BD Falcon, Franklin Lakes, NJ, USA) at 3.0 × 10^5 cells/well and cultured for 16 h. After washing with PBS, the cells were treated with 1 mL of 10% FBS-DMEM containing various concentrations of wakazu, 10 mM NaPB as negative control, or 100 ng/mL of LPS as positive control and incubated for 3 h. Total RNA was isolated from the cells using Sepasol-RNA I Super G (Nacalai Tesque) according to the

manufacturer's instructions and used as a template for cDNA synthesis with MMLV-reverse transcriptase (Promega, Madison, WI, USA) and an oligo-$(dT)_{20}$ primer (Toyobo, Osaka, Japan). A real time RT-PCR mixture, with a final volume of 20 μL, consisted of Thunderbird SYBR qPCR Mix (Toyobo), 10 pmol of a forward primer, 10 pmol of a reverse primer, and 0.1 μg of a cDNA sample. Thermal cycling conditions were 20 s at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. PCR products were measured on a StepOnePlus Real-time PCR System (Applied Biosystems, Foster City, CA, USA), and relative gene expression was calculated based on the comparative CT method using StepOne Software v2.1 (Applied Biosystems). Expression of the β-actin gene was used as an endogenous control. Specific oligonucleotide sequences for each gene are as follows. Mouse β-actin: sense, 5'-CATCCGTAAAGACCTCTATGCCAAC-3' and antisense, 5'-ATGGAGCCACCGATCCACA-3'; mouse TNF-α: sense, 5'-CTACTCCCAGGTTCTCTTCAA-3' and antisense, 5'-GCAGAGAGGAGGTTGACTTTC-3'; mouse IL-6: sense, 5'-AAGCCAGAGTCCTTCAGAGAGAT-3' and antisense, 5'-TTGGATGGTCTTGGTCCTTAGC-3'; mouse inducible nitric oxide synthase (iNOS): sense, 5'-CCAAGCCCTCACCTACTTCC-3' and antisense, 5'-CTCTGAGGGCTGACACAAGG-3'.

2.6. Griess assay

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 96-well cell culture plate at 6.0 × 10^4 cells/well and cultured for 10 h. After washing with PBS, the cells were treated with 200 μL of 10% FBS-DMEM containing various concentrations of wakazu or 10 mM NaPB as negative control and incubated for 24 h. The concentration of nitric oxide (NO) in the culture media was measured by Griess Reagent System (Promega, Tokyo, Japan) according to the manufacturer's instructions.

2.7. Western blot analysis

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 35 mm dish (BD Falcon) at 6.0 × 10^5 cells/dish at 2 mL and cultured for 16 h. After washing with PBS, the cells were treated with 2 mL of 10% FBS-DMEM containing various concentrations of wakazu, 10 mM NaPB as negative control, or 100 ng/mL of LPS as positive control and incubated for 15 min. Cytosolic and nuclear proteins were prepared using a CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich) according to the manufacturer's instructions. The protein concentration of cell lysate was determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) with BSA as a standard. Denatured proteins were then separated using SDS-PAGE and transferred onto a PVDF membrane (Hybond-P; GE Healthcare, Buckinghamshire, UK). Immunoblotting with various antibodies was performed as previously described [5].

2.8. Measurement of phagocytosis activity

RAW264.7 cells suspended in 10% FBS-DMEM were seeded into a 24-well cell culture plate at 3.0 × 10^5 cells/well and cultured for 16 h. After washing with PBS, the cells were treated with 1 mL of 10% FBS-DMEM containing various concentrations of wakazu, 10 mM NaPB as negative control, or 100 ng/mL of LPS as positive control and incubated for 6 h. After washing with PBS, 1 mL of 10% FBS-**RPMI 1640** medium containing 40 μg of Texas Red-conjugated Zymosan A (***S. cerevisiae***) BioParticles (Molecular Probes, Eugene, Ore., U.S.A.) was added to each well and incubated for 1 h under a dark condition. After removing the culture medium, the cells were collected and centrifuged at 160 x *g* for 5 min at 4°C. The cells pellet was suspended with 1 mL of 2% FBS-PBS, and phagocytotic activity was measured on a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA).

2.9. Statistical analysis

Data obtained were expressed as mean ± standard deviation. One way ANOVA followed by Tukey-Kramer test was used to assess the statistical significance of the difference. Values with $^{*}p < 0.05$ or $^{**}p < 0.01$ were considered statistically significant.

3. Results

3.1 Effect of wakazu on cytokine production by RAW264.7 cells

The effect of wakazu on cytokine production by RAW264.7 cells was first examined. Wakazu was added to the culture media at various concentrations, and the cytokine concentration in the medium was measured by ELISA after incubation for 6 h. As shown in Figure 1, wakazu significantly enhanced the production of IL-6 and TNF-α by RAW264.7 cells in a dose-dependent manner.

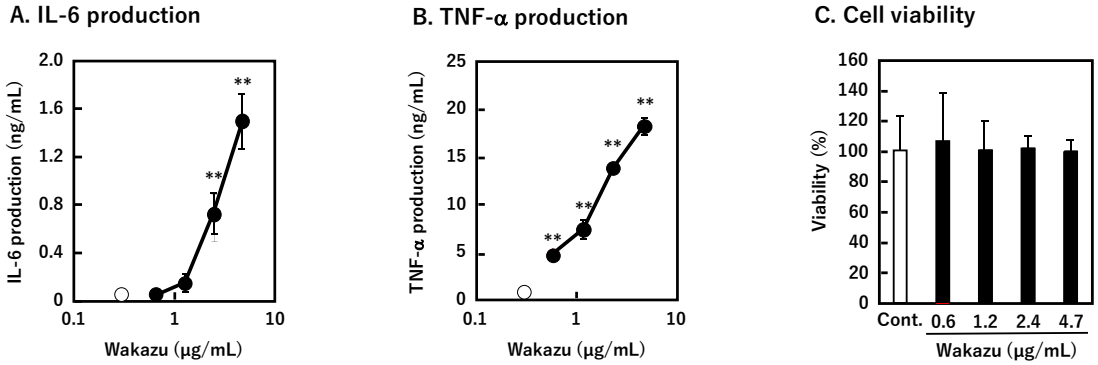

Figure 1. Effect of wakazu on cytokine production by RAW264.7 cells. (**A, B**) RAW264.7 cells were treated with 10% FBS-DMEM containing various concentrations of wakazu (gray circle) or 10 mM NaPB as control (open circle) and incubated for 6 h at 37°C. After incubation, the concentrations of IL-6 and TNF-a in the culture media were measured by ELISA. (**C**) Cell viability was measured after collecting the culture media from each well for ELISA. Experiments were performed in triplicate, and error bars indicate as the mean ± standard deviation. $^{**}p < 0.01$ against control by Tukey-Kramer test.

3.2 Effect of wakazu on cytokine gene expression levels in RAW264.7 cells

As mentioned above, wakazu stimulated the production on IL-6 and TNF-α by RAW264.7 cells. Hence, the effect of wakazu on mRNA expression levels of these cytokines was evaluated. Wakazu was added to the culture media at various concentrations, and the mRNA expression levels of IL-6 and TNF-α were evaluated by real-time RT-PCR. Wakazu significantly increased the mRNA expression levels of IL-6 and TNF-α in RAW264.7 cells. These results indicated that wakazu stimulates cytokine production by promoting the transcription process.

3.3 Effect of wakazu on NO production and mRNA expression of iNOS in RAW264.7 cells

NO is recognized as one of the most versatile compounds in the immune system, and produced by macrophages which are activated by cytokines and microbial compounds. Therefore, we examined the effect of wakazu on NO produced by RAW264.7 cells. As shown in Figure 2A, wakazu significantly stimulated NO production by RAW264.7 cells. In addition, wakazu also enhanced the mRNA expression of iNOS in RAW264.7 cells (Figure 2B), suggesting that wakazu stimulates NO production by enhancing iNOS gene expression.

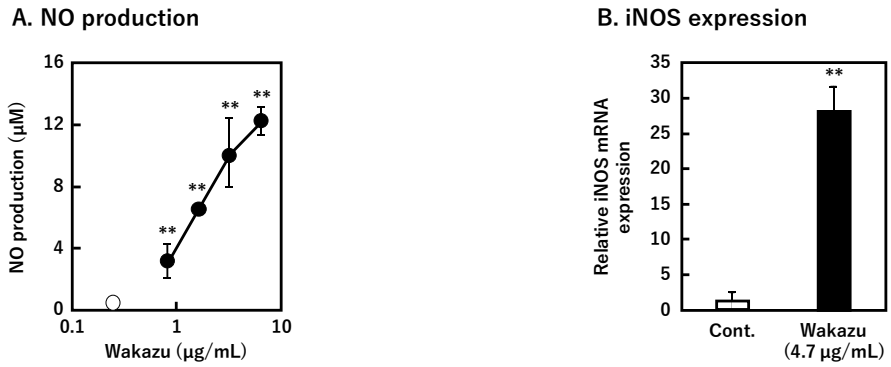

Figure 2. Effect of wakazu on NO production and mRNA expression of iNOS in RAW264.7 cells. (**A**) RAW264.7 cells were treated with 10% FBS-DMEM containing various concentrations of wakazu (gray circle) or 10 mM NaPB as control (open circle) and incubated for 24 h at 37°C. After incubation, the concentrations of NO in the culture media were measured by Griess assay. (**B**) RAW264.7 cells were treated with 10% FBS-DMEM containing 4.7 μg /mL of wakazu or 10 mM NaPB as control and incubated for 3 h. After incubation, mRNA expression levels of iNOS was evaluated by Real time RT-PCR. Experiments were performed in triplicate, and error bars indicate as the mean ± standard deviation. $^{**}p < 0.01$ against control by Tukey-Kramer test.

3.4. Effect of wakazu on NF-κB and MAP kinase signaling pathways involved in macrophage activation

To examine the involvement of NF-κB and MAP kinase signaling pathways in the regulation of the TNF-α and IL-6 production, RAW264.7 cells were incubated in the medium containing wakazu for 15 min, and the protein levels of signal molecules were evaluated by western blot analysis. The result showed that the degradation of IκBα and the translocation of NF-κB into the nucleus were stimulated by wakazu (data not shown). In addition, the phosphorylation levels of ERK and JNK were increased by wakazu, whereas that of p38 was not affected. These results suggested that wakazu enhances translocation of NF-κB to the nucleus and MAP kinase signal transduction in RAW264.7 cells.

3.5. Effect of wakazu on phagocytotic activity of RAW264.7 cells

Phagocytosis occurs in specialized cells such as macrophages, dendritic cells, and neutrophils. It is the first step in triggering host defense and inflammation. Thus, the effect of wakazu on phagocytotic activity of RAW264.7 cells was examined. The phagocytotic activity of RAW264.7 cells was obviously elevated by wakazu (data not shown), suggesting that wakazu stimulates the phagocytotic activity of macrophages.

4. Discussion

Black vinegar is produced by saccharification, fermentation, and aging in one pot. Wakazu is an immature black vinegar collected before aging for 1-3 years. Various microorganisms such as Aspergillus, yeasts, lactic acid bacteria, and acetic acid bacteria are involved in their process of fermentation and maturation. Since many of the fermenting microorganisms die in this process, wakazu contains many components derived from the microorganisms such as endotoxin which activates macrophages. Therefore, we removed the endotoxin from wakazu before evaluating the activity of wakazu and studied the immunostimulatory effect of wakazu components not derived from endotoxins. Wakazu significantly enhanced the production of IL-6 and TNF-α by RAW264.7 cells. In addition, the expression levels of IL-6 and TNF-α◎genes in RAW264.7 cells were enhanced in dose-dependent manners. These results suggest that wakazu stimulates cytokine production by upregulating the transcription process of the cytokine genes.

Next, we examined the effect of wakazu on the signaling pathways involved in macrophage activation. The activation of macrophages is caused by NF-κB and MAP kinase cascades. The result showed that the phosphorylation levels of ERK and JNK were increased by treatment with wakazu. Wakazu also helped degradation of IκBα and translocation of NF-κB into the nucleus. These results suggest that wakazu stimulates cytokine production through upregulation of the NF-κB and MAP kinase cascades. In addition, wakazu stimulated NO production and phagocytotic activity of RAW264.7 cells. So far, the NF-κB and MAP kinase cascades have been reported to be involved in NO production and the phagocytosis activity of macrophages [6,7]. Therefore, the wakazu-induced NO production and phagocytosis activity of macrophages are considered to be due to the upregulation of the NF-κB and MAP kinase cascades.

5. Conclusions

Although several function of wakazu on human health are known, the immunostimulatory effect of wakazu components on macrophages has not been reported. In this study, we found that endotoxin free-wakazu has an immunostimulatory effect on macrophages. Our findings indicate that not only kurozu, but also wakazu is also expected to be effective on our health as functional food.

References

1. Gordon, S. Pattern recognition receptors: doubling up for the innate immune respons. *Cell* **2002**, *111*, 927-930, 10.1016/S0092-8674(02)01201-1.
2. Nathan, C.F. Secretory products of macrophages. *J Clin Invest* **1987**, *79*, 319-326, 10.1172/JCI112815.
3. Nishidai, S.; Nakamura, Y.; Torikai, K.; Yamamoto, M.; Ishihara, N.; Mori, H.; Ohigashi, H. Kurozu, a traditional vinegar produced from unpolished rice, suppresses lipid peroxidation *in vitro* and in mouse skin. *Biosci Biotechnol Biochem* **2000**, *64*, 1909-1914, 10.1271/bbb.64.1909.
4. Awane, S.; Nishi, K.; Ishida, M.; Nagano, M.; Hashiguchi, K.; Fujii, A.; Sugahara, T. Inhibitory effect of Japanese black vinegar on IgE-mediated degranulation of RBL-2H3 cells and a murine model of Japanese cedar pollinosis. *Cytotechnology* **2018**, *70*, 961-974, 10.1007/s10616-018-0208-6.
5. Kumalasari, I.D.; Nishi, K.; Putra, A.B.; Sugahara, T. Activation of macrophages stimulated by bengkoang fiber extract through Toll-like receptor-4. *Food Funct* **2014**, *5*, 1403-1408, 10.1039/c3fo60360a.
6. Chen, B.-C.; Chen, Y.-H.; Lin, W.-W. Involvement of p38 mitogen-activated protein kinase in lipopolysaccharide-induced iNOS and COX-2 expression in J774 macrophages. *Immunology* **1999**, *97*, 124-129, 10.1046/j.1365-2567.1999.00747.x.
7. Blander J.M.; Medzhitov, R. Regulation of phagosome maturation by signals from Toll-Like receptors, *Science* **2004**, *304*, 1014-1018, 10.1126/science.1096158.