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Functional ingredient derived from processed brown rice extract activates human macrophage and promotes its shifting from mitochondrial to glycolytic energy metabolic activation

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ABSTRACT

Background: Brown rice (BR) has various beneficial effects. Recently, functional ingredient derived from processed BR (Kinmemai rice extract [KR-ex.]) has been developed in Japan, which reportedly decreases subjects' "susceptibility to catching colds." However, the effects of KR-ex. on the immune system remain unclear.

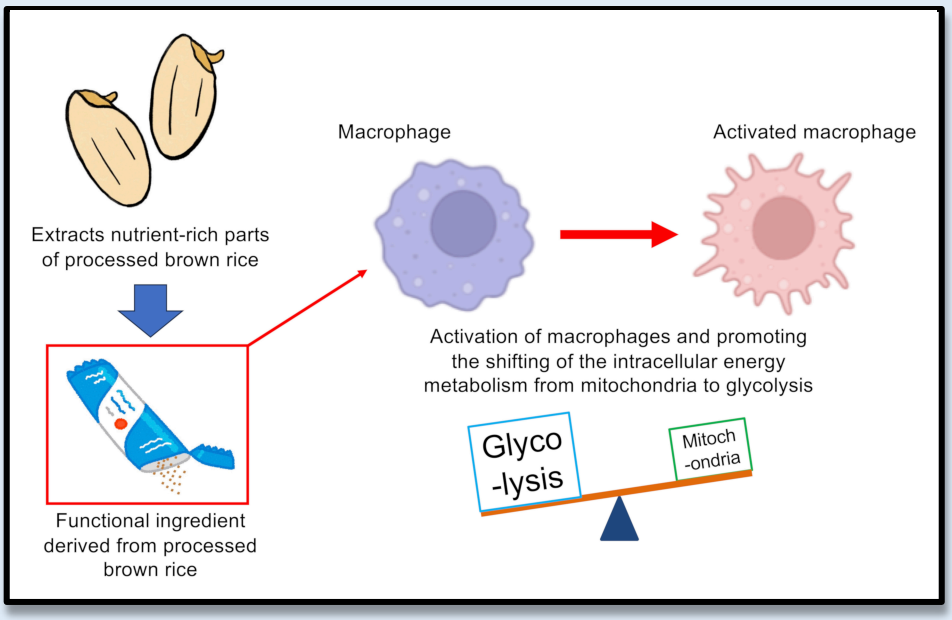
Objective: This study aimed to examine the immunostimulant effects of KR-ex., focusing on the expression of activation markers and intracellular energy metabolic activity using human monocyte (U937)-derived macrophage cells.

Methods: Cell activity was assessed using the gene expression of interleukin-1β and cyclooxygenase-2 as activation markers. The energy metabolic activity in mitochondria was evaluated using the expression of *uncoupling protein 2* and the oxygen consumption rate, whereas that in glycolysis was evaluated using the expression of *glucose transporter 1* and the amount of lactate released.

Results: The results showed that the KR-ex. group had significantly higher expression of activation markers than the control group. Moreover, the KR-ex. group showed decreased mitochondrial metabolic activity and increased glycolytic metabolic activity compared to the control group.

Conclusions: These results suggest that KR-ex. activates human macrophages and promotes the shifting of the intracellular energy metabolism from the mitochondria to glycolysis.

Keywords: Brown rice; Functional ingredient; Immunostimulant effect; Macrophage; Intracellular energy metabolism; Mitochondria; Glycolysis



INTRODUCTION

Rice, identified by its scientific name *Oryza sativa* L., is among the top three important food crops and is consumed by over half of the world's population [1,2]. In Japan, there are two main types of rice being consumed: white rice (WR) and brown rice (BR). In WR, the germ and bran layer are removed, whereas in BR, only the husk is removed; moreover, BR primarily consists of the bran layer (6%–7%), germ (2%–3%), and endosperm (90%) [3]. Because the bran layer is rich in dietary fiber, minerals, phytochemicals, and vitamin B complex, BR has many health benefits compared with WR [4-6]. BR has also been reported to have antioxidative [7-10], cholesterol-lowering [10-12], antidiabetic [13-16], anticancer [17-19], anti-inflammation [20-22], and immune activity or modulatory effects [23-27]. However, many people dislike BR because of its taste and hard texture, which are attributed to the bran layer and wax layer (outer layer of the bran layer), respectively [3,28]. To address these issues, a rice milling technology (Saika-style rice polishing process) has been developed to produce dewaxed BR (DBR, BR without the wax layer) [29], which has improved water absorption, taste, and cooking methods while retaining its nutrients [24,30]. Moreover, a new type of rice called “sub-aleurone layer residual rinse-free rice” has been produced through a special rice processing method that removes the bran layer and leaves the highly nutritious sub-aleurone layer (located between the bran layer and the starch layer), embryobase (“kinme”), and boundary with the endosperm [31]. This new type of BR, which has improved texture and taste, is known as “Kinmemai rice” in Japan.

In recent years, a functional ingredient (mature extract consisting of the sub-aleurone layer, blastema of the germ, and crushed cell group: Kinmemai rice extract [KR-ex.]) derived from BR has been developed and reported to decrease subjects' "susceptibility to catching colds" [28]. The reason for this effect may be related to the immune-active effects of BR; however, the relationship between the immunostimulant effects and KR-ex. remains unclear. If KR-ex. is verified to have an immunostimulant effect, it is expected that many people will use it as a healthy food, contributing to a healthy society.

The immune system includes both the innate and adaptive immune systems. The former is the first line of defense against various bacterial and viral infections and involves phagocytic cells such as macrophages and neutrophils [32]. Thus, the activation of the innate immune system is crucial to prevent the common cold.

Recent studies have revealed that intracellular energy metabolism in the mitochondria and glycolytic system in macrophages is closely related to its phenotype: the activity of proinflammatory macrophages (M1) rely mainly on glycolysis, whereas the activity of anti-inflammatory macrophages (M2) rely mainly on the mitochondrial electron transport chain [33]. Drawing from these previous studies, we hypothesized that owing to the resistance to colds, KR-ex. shifts the energy metabolism of macrophages to a state of high foreign body elimination capability, which increases M1 activation markers. To test this hypothesis, this study was conducted to examine whether KR-ex. has an active effect on the innate immune system, focusing on the expression of active markers and intracellular energy metabolism activity in the mitochondria and glycolysis related to the activation of macrophages using macrophage-like cells differentiated from monocyte-strain cells. In addition, the active effects of KR-ex. on macrophages were compared with those of the bran layer and endosperm.

METHODS

Cell line: The human monocyte cell line (U937 cells) was purchased from the European Collection of Authenticated Cell Cultures (Wiltshire, UK). They were maintained in RPMI-1640 medium with L-glutamine and phenol red (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (Moregate BioTech, QLD, AU), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a 5% CO_2/95% air humidified atmosphere. The cells were differentiated into macrophage-like cells using 25 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, MO, USA) for 72 h. Thereafter, they were cultured in a medium without PMA for 48 h. These methods were based on previous reports [34,35]. PMA only-stimulated macrophage is an inactive phenotype (M0) [36].

BR samples: BR samples (bran layer, KR-ex., and endosperm) were kindly provided by the Toyo Rice Corporation in crushed powder form. The bran layer and endosperm were removed during the production of KR-ex. A 3.5-g sample of KR-ex. product contains 292.3 mg of phytic acid, 7.95 mg of γ-aminobutyric acid (GABA), 6.65 mg of γ-oryzanol, and 149.1 μg (estimated value) of lipopolysaccharide (LPS) [28]. One gram of each sample was dissolved in 10 mL of distilled water, heat-treated with stirring (95°C, 1 h, 300 rpm), and then filtered through a 0.45-μm filter (100 mg/mL of each sample). These samples were additionally diluted with water/culture medium to obtain six different concentrations (3.33, 10, 33.3, 100, 333, and 1000 μg/mL).

Reagents: LPS was purchased from Macrophi Inc. (Kagawa, Japan). The Seahorse XFp Cell Mito Stress Test Kit containing oligomycin, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), and antimycin A/rotenone was purchased from Agilent Technologies (CA, USA).

Cell viability assay: U937 cells were seeded onto a 96-well plastic plate (1.25×10^4 cells/well). They were treated with 25 ng/mL of PMA using the above methods to induce differentiation into macrophage-like cells. After treatment with sterilized water (control), 1 μg/mL of LPS (positive control), or each sample of BR for 24 h, the cell viability was examined based on the increase in cytosolic lactate dehydrogenase (LDH) concentration in the supernatant using the Cytotoxicity LDH Assay Kit-WST (Dojindo Molecular Technologies Inc., Kumamoto, Japan). The assay was conducted in accordance with the protocols of the manufacturer, and the results are given as a percentage of the control.

Total RNA isolation, reverse transcription, and real-time polymerase chain reaction (PCR): The mRNA expression of interleukin (IL)-1β, cyclooxygenase (COX)-2, uncoupling protein 2 (UCP2), and glucose transporter 1 (GLUT1) was measured by quantitative real-time reverse transcription PCR (qRT-PCR) using specific primers (Table 1). Total cellular RNA was extracted and purified from U937 cells using the NucleoSpin® TriPrep kit (Macherey-Nagel, Dueren, Germany) in accordance with the manufacturer's instructions. Four hundred nanogram of total RNA was used for first-strand cDNA synthesis at a final volume of 10 μL using the PrimeScript® RT Master Mix (TaKaRa, Shiga, Japan). One microliter of cDNA (equivalent to 40 ng) was amplified by PCR using the Power SYBR® Green PCR Master Mix (Life Technologies, CA, USA) on a QuantStudio7 Real-Time PCR System (Applied Biosystems, MA, USA). The reaction protocol was as follows: 95°C for 20 s, with 45 cycles (95°C for 3 s and 60°C for 30 s). The relative gene expression levels were assessed using the ddC_t method and normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 1. Real-time PCR primers. F, forward; R, reverse.

Gene	Sequence (5'→ 3')	References
IL-1β	F: GCCATGGACAAGCTGAGGAAG R: GTGCTGATGTACCAGTTGGG	37
COX-2	F: TGCAGTGAGCGTCAGGAG R: CAAGGATTTGCTGTATGGCTGAG	38
UCP2	F: CTACAGCCAGCGCCCAGTA R: TCAGTACGCACCATGGTCAGA	39
GLUT1	F: TTGCAGGCTTCTCCAACTGGAC R: CAGAACCAGGAGCACAGTGAAG	40
GAPDH	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	41

Mitochondrial stress test: U937 cells were seeded onto an eight-well plastic plate (5.0×10^4 cells/well, Agilent Technologies) and treated with 25 ng/mL of PMA using the above methods. After treatment with sterilized water, LPS, or KR-ex. for 24 h, the culture medium was removed and substituted with the XF RPMI Medium without phenol red (Agilent Technologies) comprising 10-mM glucose, 1-mM pyruvate, and 2-mM glutamine. Subsequently, the cellswere incubated for 1 h at 37°C without CO_2. Next, 15-μM oligomycin (1.5 μM final), 5-μM FCCP (0.5 μM final), and 5-μM antimycin A/rotenone (0.5 μM final) were loaded into injection ports A, B, and C of the XFp Sensor Cartridge (Agilent Technologies), respectively, in accordance with the manufacturer's protocols. The oxygen consumption rate (OCR) was measured with the XF HS Mini Analyzer (Agilent Technologies) using the Mito Stress Test Kit standard protocol.

Lactate release assay: U937 cells were seeded onto a 12-well plastic plate (1×10^6 cells/well) and treated with 25 ng/mL of PMA using the above methods. After treatment with sterilized water, LPS, or KR-ex. for 24 h, the supernatants were collected. The amount of lactate in the supernatants was measured using the Lactate Assay Kit-WST (Dojindo Molecular Technologies Inc.) in accordance with the manufacturer's recommended protocols.

Statistical analysis: All experiments were replicated at least three times. All data are presented as mean ± standard deviation of the mean. The cell viability and expression of activation markers (*IL-1β*, *COX-2*) for determining the experimental concentrations of KR-ex. were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Comparisons of the expression of activation markers, *UCP2*, *GLUT1*, and lactate release assay among BR samples were analyzed using one-way ANOVA followed by Bonferroni post hoc test. The Mito Stress Test was assessed using Student's *t*-test between two groups. *P*-values of 0.05, 0.01, or 0.001 were considered to denote a statistically significant difference. All statistical analyses were performed using R statistical software version 4.2.2.

RESULTS

Determination of KR-ex. experimental concentrations: The cytotoxic concentration of KR-ex. was first determined using cell viability assay to determine the experimental concentration of KR-ex. Figure 1a shows no differences in cell viability at 3.33–1000 μg/mL of KR-ex. compared with control.

Subsequently, the mRNA expression of IL-1β and COX-2 as activation markers of macrophages was examined to assess the stimulatory effects of KR-ex. to macrophage-like cells at each concentration. Figures 1b and c show that the expression of activation marker genes was significantly higher in LPS-treated macrophage-like cells (LPS group) than in the control group. Moreover, the expression of these genes in the KR-ex. group increased in a concentration-dependent manner. Specifically, the expression of *IL-1β* at ≥100 μg/mL and *COX-2* at ≥333 μg/mL of KR-ex. was significantly higher than that of the control.

Based on these results, the following experimental concentrations of KR-ex. were used: 100, 333, and 1000 μg/mL.

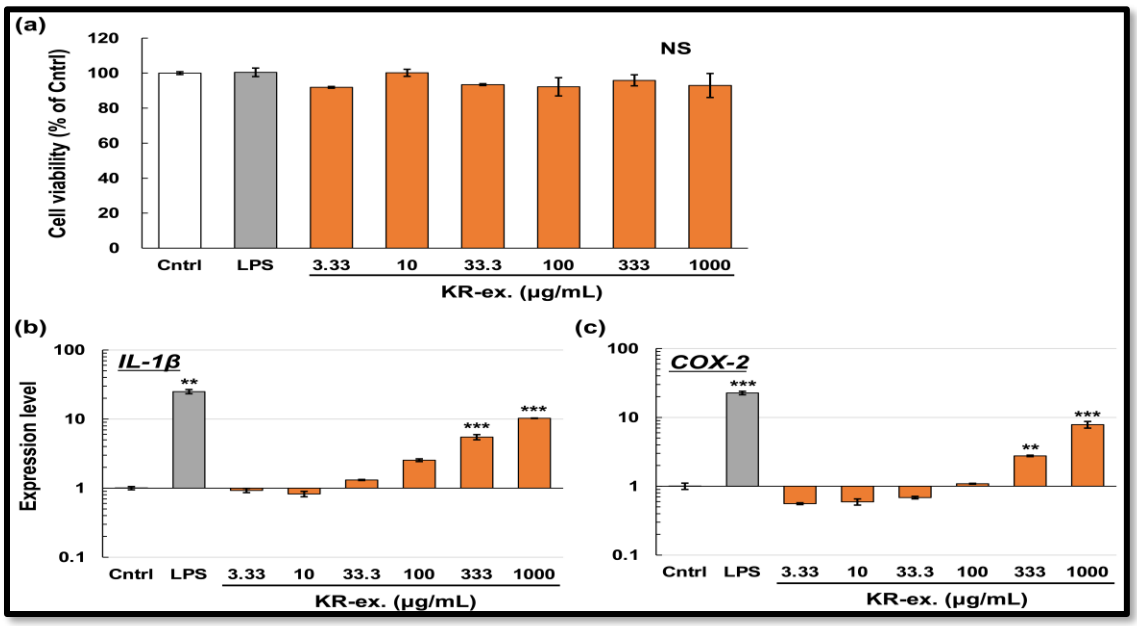


Figure 1. Determination of the experimental concentration of Kinmemai rice extract (KR-ex.). (a) U937-derived macrophages were incubated for 24 h after treatment with sterilized water, LPS, or KR-ex., and the cell viability was assessed using the LDH release assay (n = 4). (b, c) The cells were treated with LPS or KR-ex. for 24 h, and total RNA was then collected. The gene expression levels of IL-1β and COX-2 were analyzed using real-time PCR and expressed as ΔΔCt (n = 3). Cntrl denotes cells treated with sterilized water; LPS denotes cells treated with 1 μg/mL of LPS; KR-ex. denotes cells treated with each concentration of KR-ex. NS, not significant; $^{**}p < .01$; $^{***}p < .001$.

Comparison of the immunostimulatory effects among BR samples: The gene expression of macrophage activation markers was examined to compare differences in immunostimulatory effects among BR samples. Figure 2a shows no differences in cell viability at 333–1000 μg/mL of bran layer or endosperm compared with the control. Similar to the KR-ex. group, the expression of activation markers in the bran layer group increased in a concentration-dependent manner. However, the bran layer group had slightly lower expression levels than the KR-ex. group (Figures 2b and c).

Alternatively, the expression levels in the endosperm group were significantly lower than those in the bran layer and KR-ex. groups at ≥333 μg/mL. Moreover, there were no observed significant differences in *IL-1β* and *COX*-2 expression between the endosperm group and control group.

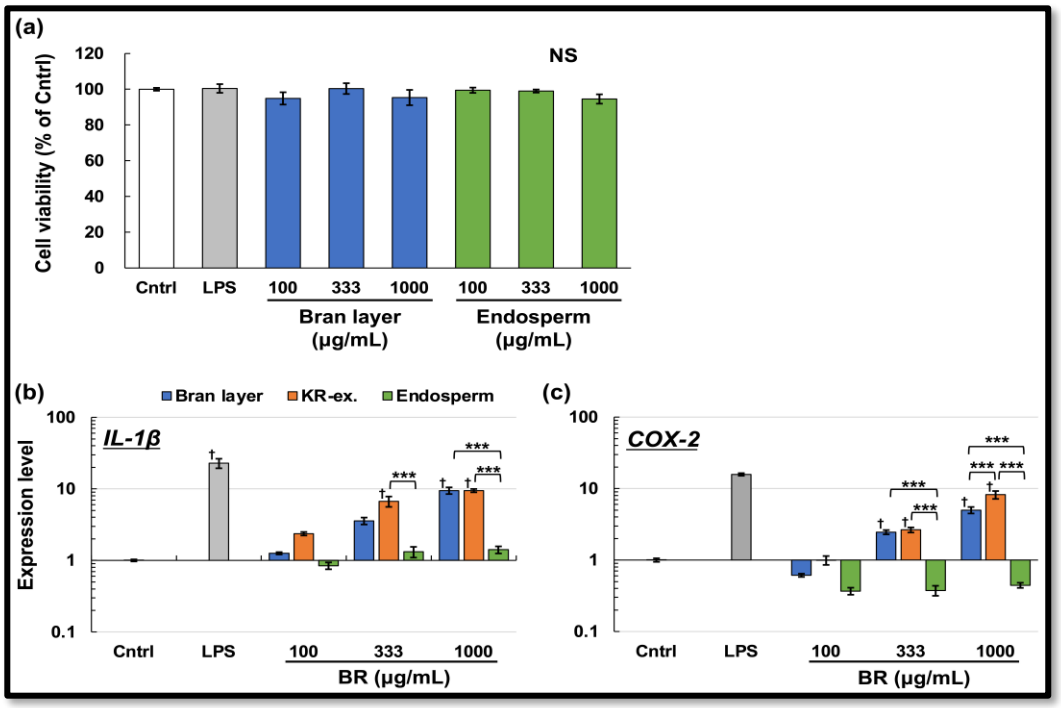


Figure 2. Comparison of the active effects on macrophages among brown rice (BR) samples. (a) U937-derived macrophages were incubated for 24 h after treatment with sterilized water, LPS, bran layer, or endosperm, and cell viability was assessed using the LDH release assay (n = 4). (b, c) The cells were treated with LPS or BR samples for 24 h, and total RNA was then collected. The gene expression levels of IL-1β and COX-2 were analyzed using real-time PCR and expressed as ΔΔCt (n = 3). Cntrl denotes cells treated with sterilized water; LPS denotes cells treated with 1 μg/mL of LPS; BR denotes cells treated with each BR sample. NS, not significant; ***$p < .001$; $^{\dagger}p < .05$ vs. Cntrl.

Expression of genes related to intracellular energy metabolism in BR-treated cells: Figure 3a shows that the expression of *UCP2* was significantly lower in the LPS or BR sample groups than in the control group. In particular, the bran layer or KR-ex. group showed significantly decreased expression of *UCP2* compared with the endosperm group. By contrast, the LPS group had increased expression of *GLUT1* compared with the control group. However, no significant differences were observed (Figure 3b). The expression of *GULT1* in the BR sample groups was significantly higher than that in the control group, and the KR-ex. group showed the highest expression level of *GLUT1.*

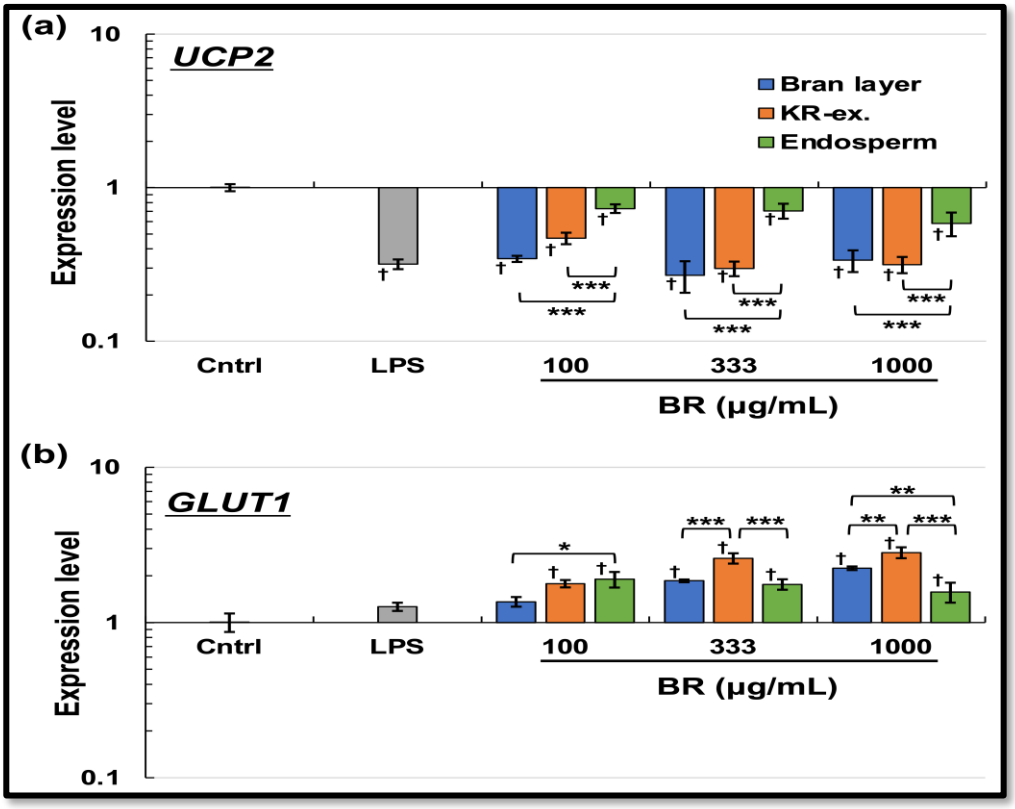


Figure 3. Mitochondrial and glycolytic energy metabolism-related genes expression in macrophages treated with each BR sample. U937-derived macrophages were incubated for 24 h after treatment with sterilized water, LPS, or each BR sample, and total RNA was then collected. The gene expression levels of (a) uncoupling protein 2 (UCP2) and (b) glucose transporter 1 (GLUT1) were analyzed using real-time PCR and expressed as ΔΔCt. Cntrl denotes cells treated with sterilized water; LPS denotes cells treated with 1 μg/mL of LPS; BR (Brown Rice) denotes cells treated with each BR sample. n = 3; *p < .05; **p < .01; ***p < .001; $^{\dagger}p$ < .05 vs. Cntrl.

Effects of KR-ex. on mitochondrial and glycolytic metabolism in macrophage-like cells: The OCR and amount of lactate released were measured using the Mito Stress Test and lactate release assay, respectively, to examine the effects of KR-ex. on mitochondria and glycolytic energy metabolism.

Figure 4a shows no differences in basal and maximum respiration between the LPS group and the control group. However, the LPS group tended to show decreased basal and maximum respiration compared with the control group. By contrast, the KR-ex. group showed significantly decreased basal and maximum respiration compared with the control group (Figure 4b). On the other hand, the amount of lactate released from cells treated with LPS or KR-ex. was significantly higher than that of the control (Figure 5).

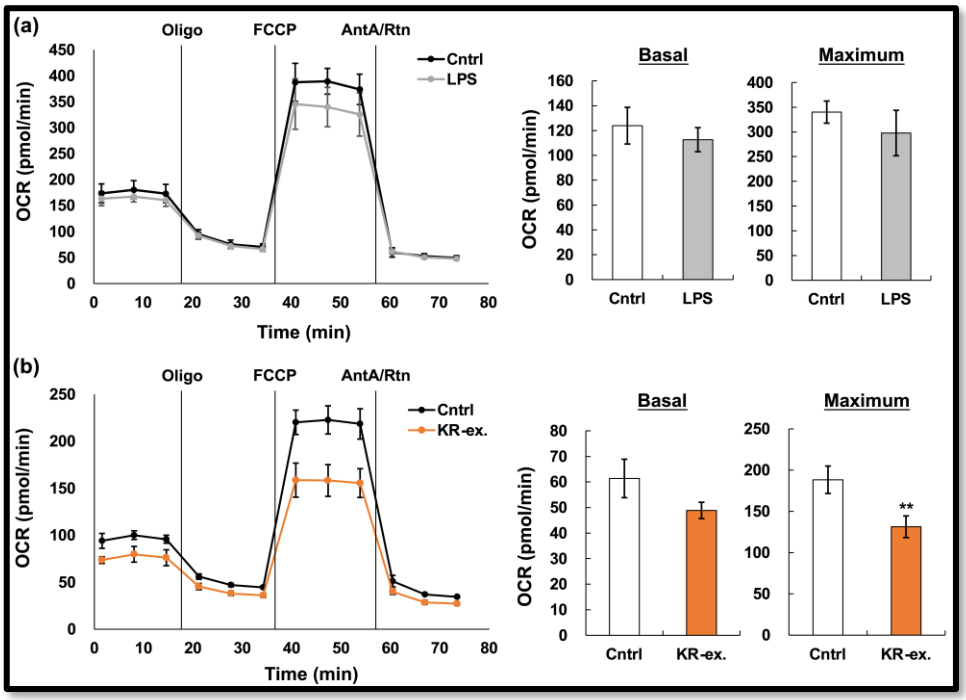


Figure 4. Assessment of mitochondrial energy metabolic activity using Mito Stress Test. U937-derived macrophages were incubated for 24 h after treatment with sterilized water, LPS, or KR-ex. The oxygen consumption rates (OCRs) of samples were measured using the XF HS Mini Analyzer. Final concentration of each reagent: 1.5-μmol/L oligomycin (Oligo), 0.5-μmol/L carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), and 1-μmol/L antimycin A and rotenone (AntA/Rtn). Respiratory parameters: Basal and Maximum denote basal respiration and maximum respiration, respectively. (a) Comparison between the Cntrl and LPS group. (b) Comparison between the Cntrl and KR-ex. group. Cntrl denotes cells treated with sterilized water; LPS denotes cells treated with 1 μg/mL of LPS; KR-ex. denotes cells treated with 1000 μg/mL of KR-ex. n = 3; **p* < 0.05.

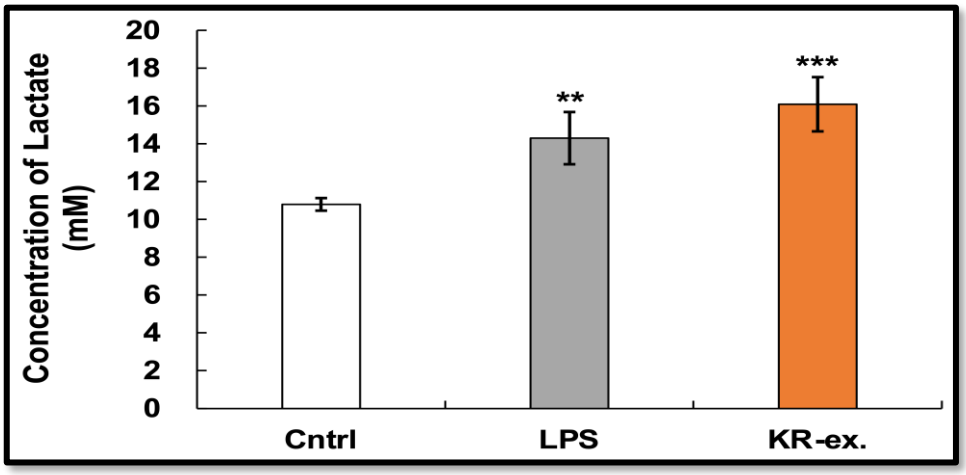


Figure 5. Assessment of glycolytic energy metabolic activity using lactate release assay**.** U937-derived macrophages were incubated for 24 h after treatment with sterilized water, LPS, or KR-ex. The amount of lactate released from samples was quantified by measuring the absorbance at 490 nm using the lactate release assay kit. Cntrl denotes cells treated with sterilized water; LPS denotes cells treated with 1 μg/mL of LPS; KR-ex. denotes cells treated with 1000 μg/mL of KR-ex. n = 3; ***p* < .01, ****p* < .001.

DISCUSSION

This study examined whether a functional ingredient derived from KR-ex. activates human macrophage-like cells, focusing on the expression of macrophage activation markers and the effects on intracellular energy metabolism between mitochondria and glycolysis.

In the KR-ex. group, the expression of macrophage activation marker genes (IL-1β and COX-2) increased in a concentration-dependent manner (Figure 1). It was assumed that 333 μg/mL of KR-ex., which significantly activates macrophage cells, contains approximately 14.19 ng of LPS because KR-ex. contains 149.1 μg of LPS in each packet (3.5 g, estimated value) [28]. Previous studies have reported that LPS extracted from various plants activates macrophages [24, 42,43]. Inagawa et al. reported that DBR extracted using hot water contains LPS, which activates RAW264.7 cells (mouse macrophage-like cell line) via Toll-like receptor 4. They further demonstrated that more than 1 mg/mL of DBR (equivalent to approximately 6.4 ng/mL of LPS) increased nitric oxide (NO) production in macrophage cells [24]. These studies suggest that LPS is mainly responsible for the active effect of KR-ex. on human macrophage-like cells.

In addition, the expression of activation markers among BR samples (bran layer, KR-ex., and endosperm) was compared to examine differences in the active effects on macrophages. The bran layer has been reported to be rich in LPS [44]. However, polished rice extracts have very low LPS content (0.037 μg/g), and NO production could not be detected in macrophages [24]. Figures 2b and c show that the bran layer removed from Kinmemai rice had increased expression of activation markers, but not the endosperm. These results are consistent with those of previous studies.

Subsequently, the intracellular energy metabolic activity between mitochondria and glycolysis was examined to investigate the active effects of KR-ex. on macrophages in more detail. There are two subtypes of macrophages: M1 and M2. M1 macrophages are activated by interferon-γ or LPS stimulation and initiate innate immunity by releasing proinflammatory cytokines. By contrast, M2 macrophages are activated through exposure to certain cytokines (e.g., IL-4 or IL-10) and suppress immune response [45]. Therefore, the PMA-stimulated macrophages in the present study fall into the M1 type. M1 macrophages was reported to rely mainly on glycolysis and impair the tricarboxylic acid cycle and mitochondrial oxidative phosphorylation [34]. UCP2 is a mitochondrial anion carrier protein that is crucially involved in energy metabolic regulation in various cells [46-48]. GLUT1 mainly contributes to basal glucose uptake and enhances glycolysis [49,50], which is crucial in energy metabolism and is related to the metabolic reprogramming of macrophages [51,52]. Thus, the expression levels of UCP2 and GLUT1 genes were assessed to examine the activity of mitochondrial and glycolytic energy metabolism among the groups, respectively.

Figure 3 shows that the expression of *GLUT1* increased in a concentration-dependent manner in the bran layer and KR-ex. groups, whereas the expression of *UCP2* was low (plateau at 333 mg/mL). Hence, these results suggest that the bran layer and KR-ex. may activate macrophages by shifting intracellular energy metabolic activity from mitochondria to glycolysis. Moreover, the down-expression level of *UCP2* in the bran layer and KR-ex. groups were the same, whereas the expression level of *GLUT1* in the KR-ex. group was greater than that in the bran layer group. These results were one of the reasons why the KR-ex. group had higher expression levels of activation markers than the bran layer group (Figure 2).

In addition, the expression of *GLUT1* in the endosperm group tended to be higher than that in the LPS group. Dey et al. reported that high-glucose conditions induced GLUT1 protein expression on the membrane of renal cells via microRNA-21 expression [53]. Thus, the glucose in the endosperm may be one of the reasons that increased *GLUT1* expression.

The Mito Stress Test and lactate release assay were performed to further examine the energy metabolic activity of 1000 μg/mL of KR-ex. with the lowest expression of *UCP2* and the highest expression of *GLUT1*. The OCRs in the KR-ex. group were significantly decreased compared with those in the control group (Figure 4a). In addition, the OCR in LPS-treated cells tended to be lower than that in the control group, although there were no significant differences (Figure 4b). Moreover, the amount of lactate released in the KR-ex. group was significantly higher than that in the control group and tended to be higher than that in the LPS group (Figure 5). These results suggest that KR-ex. efficiently shifts energy metabolic activity from mitochondria to glycolysis in macrophages. However, these findings are not in agreement with the results, where the gene expression of activation markers was lower in the KR-ex. group than in the LPS group (Figures 1b and c). Various components in BR have been reported to inhibit the production of inflammatory factors in LPS-stimulated macrophages, and these anti-inflammatory effects are caused by γ-oryzanol, phenolics, and GABA in BR [22, 54-56]. Based on these findings, in macrophages without LPS stimulation, various components in KR-ex. may efficiently shift energy metabolic activity to glycolysis while regulating it so that it does not induce excessive expression of inflammatory factors. In other words, KR-ex. may put macrophages in a state of readiness for activity so that they can be activated quickly (a primed state). To prove this hypothesis, the effects of KR-ex. on anti-inflammatory profiles and energy metabolic activity in LPS-stimulated macrophages should be examined. Moreover, studies using samples that have had the endogenous LPS in KR-ex removed or LPS-unresponsive macrophages should be conducted in the future. Because *in vitro* experiments to verify direct effects have limited ability to validate the effects of KR-ex on the immune system of complex organisms, future clinical studies *in vivo* using human blood samples are required to better demonstrate the effects of KR-ex.

CONCLUSIONS

Kinmemai rice extract can activate human macrophage-like cells and promote shifting intracellular energy metabolism from mitochondria to glycolysis, these findings support our hypothesis. Moreover, Kinmemai rice extract exhibit greater effects compared with the bran layer or endosperm. The findings may explain why Kinmemai rice extract can decrease subjects' "susceptibility to catching colds." This study may enable consumers to consume functional ingredients such as brown rice with more confidence and peace of mind. Our research is expected to contribute to the extension of healthy life expectancy and further reduction of medical costs by improving health through the active consumption of Kinmemai rice extract by each individual.

List of abbreviations: BR, brown rice; COX-2, cyclooxygenase-2; DBR, dewaxed brown rice; GABA, γ-aminobutyric acid; GLUT1, glucose transporter 1; IL-1β, interleukin-1β; KR-ex., Kinmemai rice extract; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; OCR, oxygen consumption rate; PMA, phorbol 12-myristate 13-acetate; UCP2, uncoupling protein 2

Competing interests: The authors declare no conflict of interest.

Authors' contributions: [K.T.]: [Conceived and designed the experiments], [Performed the experiments], [Analyzed the data], [Contributed to scientific discussions], [Wrote the paper]; [K.D.]: [Conceived and designed the experiments], [Performed the experiments], [Analyzed the data], [Contributed to scientific discussions], [Revised the paper]; [T.S.]: [Conceived and designed the experiments], [Contributed to scientific discussions], [Revised the paper]; [K.W.]: [Conceived and designed the experiments], [Contributed to scientific discussions], [Revised the paper]; [Y.J.]: [Contributed to scientific discussions], [Revised the

paper]; [K.T. (Koji Tomobe)]: [Contributed to scientific discussions], [Revised the paper]. All authors have read and approved the final version of the manuscript.

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