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RESEARCH ARTICLE

Black Rice Extract Reduces Body Weight, Waist Circumference, Body Mass Index and Lipopolysaccharide in Obese Subjects: A Preliminary StudyArmanto Makmun^{1,*}, Agussalim Bukhari², Nurpudji Astuti Taslim², Aminuddin², Ferry Sandra^{3,4}¹Faculty of Medicine, Moslem Universitas Muslim Indonesia, Jl. Urip Sumoharjo Km.5, Makassar 90231, Indonesia²Department of Nutrition, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan Km.10, Makassar 90245, Indonesia³Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia⁴Center of Molecular Biology Study, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia

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Abstract

BACKGROUND: The prevalence of obesity, or an excessive fat accumulation, is keep increasing. In obesity, inflammation can be induced by leaky gut due to the intestinal tight junction barrier dysfunction. Zonula occludens-1 (ZO-1) plays a role in developing intestinal tight junction barrier dysfunction and gut microbiota imbalance, thus promote the translocation of bacterial endotoxin characterized by lipopolysaccharide (LPS) into circulation. Black rice extract (BRE) has been known to have anti-inflammatory property. This study was conducted to investigate the effect of BRE on body weight (BW), waist circumference (WC), body mass index (BMI), ZO-1 and LPS of obese patients.

METHODS: Twenty-three male subjects were divided into non-obese group (NOG), obese group (COG) and BRE-obese group (BOG). Subjects in BOG received a daily dose of 5.6 g/day BRE for 4 weeks. BW, WC and BMI, serum ZO-1 and LPS were measured before and after treatment.

RESULTS: BRE was prepared successfully and free from microbial contamination. Treatment of BRE for 4 weeks reduce BW (95.40±5.78 vs. 94.59±6.00 kg, $p=0.043$), WC (109.25±3.55 vs. 107.50±3.46 cm, $p=0.000$) BMI (32.65±1.86 vs. 32.18±1.80, $p=0.000$) and LPS (222.27±38.63 vs. 131.63±9.70 ng/mL, $p=0.020$) of obese subjects. The pre-post ZO-1 levels in all groups were not significantly different ($p>0.05$).

CONCLUSION: Treatment of 5.6 gr BRE daily for four weeks can reduce BW, WC, BMI and serum LPS, but not serum ZO-1 in obese patients. Therefore, BRE may reduce inflammation in obesity.

KEYWORDS: black rice, obesity, BW, WC, BMI, LPS, ZO-1

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Introduction

Prevalence of obesity keep increasing rapidly, it was estimated that more than one billion people in the world are now living with obesity, nearly 880 million adults and 159 million children and adolescents aged 5-19 years, and about four million people die every year due to obesity and its comorbidities.(1-4) Obesity is an abnormal or

excessive fat accumulation that may impair health due to an energy imbalance between calories consumed and calories expended.(5,6) Obesity is characterized by the increase of pro-inflammatory cytokines released from adipose tissue and the infiltration of leukocytes, especially macrophages, leading to chronic low-grade inflammation.(7,8)

Obesity has been associated with gut microbiota composition changes. One of the changes is an increase in Firmicutes and a decrease in Bacteroidetes, which will

contribute to the development of pro-inflammatory status in obesity through alteration in the intestinal barrier.(9) Zonula Occludens-1 (ZO-1) has been known to link tight junction proteins with the cytoskeleton and to provide integrity of the paracellular barrier, hence ZO-1 has been used as a biomarker of intestinal barrier integrity.(10,11) When the intestinal barrier was dysfunction, an endotoxin called lipopolysaccharide (LPS) could be transported into circulation. LPS has been reported to increased pro-inflammatory cytokines (12), therefore, the circulatory-transported LPS will cause metabolic endotoxemia and the production of pro-inflammatory cytokines leading to the development of chronic low-grade inflammation.(9)

Black rice is one variant of rice which has black pigment containing anthocyanins.(13) Compared with white rice, black rice has an abundance of phenolic compounds, which are associated with antioxidant activity. Black rice extract (BRE) was reported to have an anti-inflammatory effect on the splenocytes of a diabetes mellitus mouse model. (14) Another study also indicated that supplementation of BRE for 12 weeks had an effectiveness in reducing fat accumulation in postmenopausal women aged between 45 and 69 years.(15) Although the effects of BRE on oxidative stress and inflammation (16,17), hyperlipidaemia and hyperglycemia (18,19), body weight gain (20), lipid accumulation (21), and gut microbiota (22) have been elucidated, to our knowledge, the effect of BRE on intestinal barrier dysfunction and metabolic endotoxemia in subjects with obesity has not been clearly understood. Therefore, present study was conducted to investigate the effectiveness of BRE on ZO-1 and LPS in subjects with obesity.

Methods

Production of BRE Solution

From Toraja, South Sulawesi, 20 kg of Black rice (*Oryza sativa* L.) was obtained. The rice was milled into powder, macerated with 32 L of 70% ethanol, sonicated for 30 min, and left overnight. The next day, the solution was filtered, evaporated at 40°C, and dried at 60°C. Resulted paste was weighted, solubilized in sodium carboxymethylcellulose (Na-CMC), added with 0.5% citric acid to reach pH=3, and finally added with sorbitol to sweeten the solution.

Microbial Contamination Test

BRE solution was tested for possible contamination of microorganism with Total Plate Count (TPC) Analysis. Briefly, BRE was serial-diluted, poured and spread evenly

on Plate Count Agar (PCA), then incubated in an incubator at 37°C for 24 hours. After incubation, the formed colonies were counted.

For *Staphylococcus aureus* and *Salmonella* sp. tests, BRE solution was serial-diluted, spread evenly on Baird-Parker Agar (BPA) for *S. aureus* while Xylose Lysine Deoxycholate (XLD) Agar for *Salmonella* sp. Then the agar was incubated in an incubator at 37°C for 24 hours. After incubation, the formed colonies were counted.

Subject Recruitment and Criteria

Male subjects with age of 18-35 years old were recruited during the period of April-March 2021 at Hasanuddin University Medical Research Center (HUMRC) and at Ibnu Sina Hospital. Subjects with history of smoking, strict diet; chronic metabolic disorders (diabetes mellitus, hypertension, systemic lupus erythematosus, and rheumatoid arthritis) were excluded. Prior to the enrolment, all subject was informed and asked for their willingness to participate by signing a written informed consent form. This research protocol was approved by the Ethics Committee of the Faculty of Medicine, Hasanuddin University, Makassar (No. 300/UN4.6.4.5.31/PP36/2020). This study has been registered at clinicaltrials.gov under the registration number NCT04827628.

Anthropometric Measurement

Body weight (BW) was measured in kilogram (kg), body height was measured in centimetre (cm), waist circumference (WC) was measured in the halfway between subjects' lowest rib and the top of the hipbone, Body Mass Index (BMI) was calculated as weight (kg) divided by height squared (m²). BMI score was used to differentiate between normal weight (18.5–22.9), overweight (23–24.9), or obesity (≥25).

Subject Intervention and Sample Collection

Subjects were divided into 3 groups: non-obese group (NOG), obese group (OG), and BRE-treated obese group (BOG) for 4 weeks. Serum ZO-1 and LPS was conducted before and after treatment with BRE. After overnight fasting, 5 mL venous blood was drawn, left at room temperature for 15 minutes, then centrifuged at 3000 rpm for 15 min. Afterward, the serum was collected, aliquoted and stored at -80°C for Enzyme-linked Immunosorbent Assay (ELISA) quantifications.

ELISA for ZO-1 and LPS

Collected serum was used to determine ZO-1 and LPS levels using Human Tight Junction Protein 1 (ZO-1) ELISA Kit

(Cat No. MBS2605490; MyBioSource, San Diego, CA, USA) and Human Lipopolysaccharides (LPS) ELISA Kit (Cat No. MBS266722; MyBioSource). Both kits utilized the double antibody sandwich ELISA technique. Anti-Human ZO-1 monoclonal antibody or anti-Human LPS monoclonal antibody was the precoated antibody, while a biotinylated polyclonal antibody was used as the detection antibody. The TMB that was used as the substrate, was reacted to form a blue product and finally turns to yellow after addition of the stop solution. For obtaining optical density (OD), microplate reader was set at 450nm. ZO-1 ELISA kit could detect at the range of 1.56-100 ng/mL with sensitivity of 0.5 ng/mL, while LPS ELISA kit could detect at the range of 15.6-1,000 ng/mL with sensitivity of 5 ng/mL.

Results

The BRE solution in concentration of 93.33 mg/mL and total volume of 28 L was prepared successfully. For the microbial test results, TPC for BRE was 4.6×10^3 CFU/g, while *S. aureus* and *Salmonella* sp. counts were both negative per 0.1 g of sample.

Forty male subjects were included in the study. Based on the BMI, 15 subjects were non-obese (included in NOG) and 25 subjects were obese. The obese subjects were then divided randomly into 2 groups: 12 subjects in OG and 13 subjects in BOG. Subjects in BOG consumed 60 mL BRE solution containing 5.6 g BRE daily for 4 weeks.

However, not all subjects could complete the study, 8 subjects in NOG, 4 subjects in OG and 5 subjects in BOG were dropped out due to their health conditions during the Coronavirus Disease 2019 (COVID-19) pandemics. Therefore, in the end of the of the study there were 7 subjects in NOG, 8 subjects in OG, and 8 subjects in BOG that completed the study and assessments. All subjects of all groups had similar age ($p=0.382$, Kruskal Wallis), for NOG 21.60 ± 0.61 years old, for OG 20.13 ± 0.91 years old and for BOG 22.33 ± 0.49 years old.

BRE reduced BW, WC and BMI

In the pre-treatment stage, subjects in OG and BOG had similar BW, WC and BMI, but higher than NOG. Treatment of BRE for 4 weeks could reduce significantly the BW, WC and BMI of obese subjects, as shown in the BOG (Table 1). The BW, WC and BMI of all groups were analysed further by calculating the pre-post differences (Δ) of each group (Figure 1). All Δ BW, Δ WC and Δ BMI showed significant differences between OG and BOG, suggesting that BRE could certainly reduce BW, WC and BMI of obese subjects.

BRE reduced LPS, but did not affect ZO-1

Similar to BW, WC and BMI, in the pre-treatment stage, subjects in OG and BOG had similar LPS level, but higher than NOG. Treatment of BRE for 4 weeks could also reduce significantly the LPS level of obese subjects, as shown in the BOG (Table 2). The ZO-1 level in NOG was higher than the one in OG and BOG. The pre-post ZO-1 levels in all groups were not significantly different.

Discussion

The current study showed that four weeks of BRE consumption can significantly reduce BW, WC, BMI and LPS, but not ZO-1 level. It has been widely reported that obesity is related with chronic inflammation, which is marked by LPS in the present study. The LPS are cellular wall components of gram-negative bacteria that contain a pathogen-associated molecular pattern, Lipid A, able to interact with the toll-like Receptor 4 via the myeloid differentiation primary response 88 protein. This interaction results in the activation of the pathway downstream and nuclear factor (NF)- κ B translocation, thus increasing the gene transcription of cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, and IL-6.(23) Normally, LPS concentrations are highest in the gut lumen and low or undetectable in the circulating plasma because LPS in the gut lumen do not penetrate the healthy intestinal epithelium.(24) BRE was known to contain high level

Table 1. Pre-post BW, WC and BMI of NOG, OG and BOG groups.

Parameter	NOG (n=7)			OG (n=8)			BOG (n=8)		
	Pre	Post	p-value	Pre	Post	p-value	Pre	Post	p-value
BW (kg)	60.39 \pm 3.04	60.33 \pm 3.03	0.103 [‡]	99.83 \pm 5.47	99.99 \pm 5.42	0.135 [‡]	95.40 \pm 5.78	94.59 \pm 6.00	0.043 ^{‡*}
WC (cm)	77.71 \pm 2.83	77.76 \pm 2.85	0.180 [#]	112.75 \pm 4.06	112.85 \pm 4.08	0.291 [#]	109.25 \pm 3.55	107.50 \pm 3.46	0.000 ^{‡*}
BMI	21.84 \pm 0.75	21.86 \pm 0.78	0.736 [‡]	34.08 \pm 1.58	33.96 \pm 1.65	0.831 [#]	32.65 \pm 1.86	32.18 \pm 1.80	0.000 ^{‡*}

Data are presented in mean \pm SEM. [‡]Paired-Samples T Test; [#]Wilcoxon Signed Rank Test; ^{*}significant with $p<0.05$.

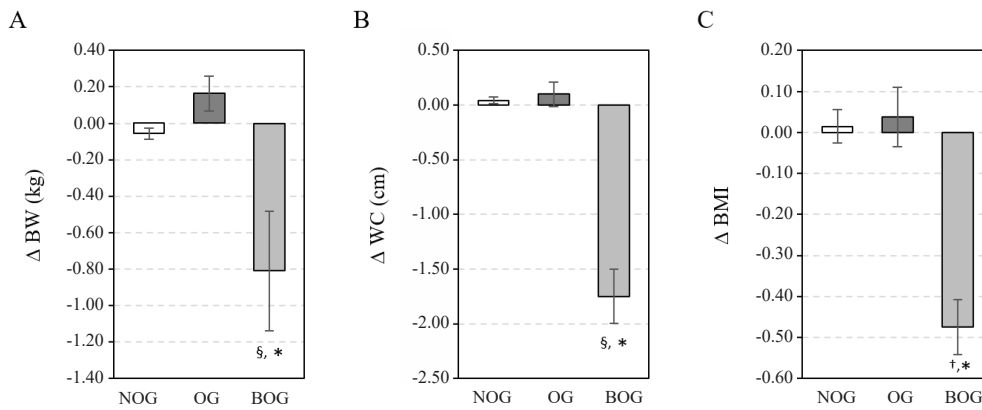


Figure 1. The Pre-post differences of BM, WC and BMI. Data of Table 1 was used to calculate the differences of pre-post of NOG, OG and BOG (mean±SEM). Δ: Pre-post difference. §Independent Samples T Test (compared with NOG); †Mann-Whitney Test (compared with NOG); *significant with $p<0.05$.

of anthocyanin. The anthocyanin in BRE, which has an anti-inflammatory effect, can modulate I-kappa-B-alpha (IκB-α) phosphorylation leading to lower expression of pro-inflammatory cytokines such as TNF-α, interferon (IFN)-γ, and ILs.(13)

It has been reported that pro-inflammatory cytokines regulated the tight junction protein ZO-1 expression.(10) Previous study reported also that high-fat diet feeding in mice could reduce the expression of ZO-1 in the jejunum. (25) In this study, the LPS level was reduced by BRE, however the ZO-1 level was not affected. Therefore, based on our present data, we suggested that the ZO-1 levels might not be detected well in the circulation. However, further larger cohort research is needed to clarify this issue.

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Authors Contribution

AM and AB were involved in the conceptualization of the study, preparation of methodology, and the investigation. AM prepared the study resources and drafted the original manuscript. NAT and A gave critical suggestions. AM and FS performed the data analysis, prepared the visualization of the data, as well as revised and edited the manuscript,

Conclusion

Consumption of 5.6 gr BRE daily for four weeks can reduce BW, WC, BMI and serum LPS, but not serum ZO-1 in obese patients. Therefore, BRE may reduce inflammation in obesity.

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Table 2. Pre-post ZO-1 and LPS levels of NOG, OG and BOG groups.

Parameter	NOG (n=7)			OG (n=8)			BOG (n=8)		
	Pre	Post	p-value	Pre	Post	p-value	Pre	Post	p-value
ZO-1 (ng/mL)	17.26±2.09	17.55±2.97	0.932 [‡]	14.27±2.06	14.63±1.95	0.901 [‡]	14.62±2.77	14.41±1.25	0.954 [‡]
LPS (ng/mL)	149.00±20.83	139.82±14.35	0.778 [‡]	214.26±41.48	206.04±25.58	1.000 [#]	222.27±38.63	131.63±9.70	0.020 ^{‡*}

Data are presented in mean±SEM. [‡]Paired-Samples T Test; [#]Wilcoxon Signed Rank Test; *significant with $p<0.05$.

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RESEARCH ARTICLE

Black Rice Extract Reduces Body Weight, Waist Circumference, Body Mass Index and Lipopolysaccharide in Obese Subjects: A Preliminary Study

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Abstract

BACKGROUND: The prevalence of obesity, or an excessive fat accumulation, is keep increasing. In obesity, inflammation can be induced by leaky gut due to the intestinal tight junction barrier dysfunction. Zonula occludens-1 (ZO-1) plays a role in developing intestinal tight junction barrier dysfunction and gut microbiota imbalance, thus promote the translocation of bacterial endotoxin characterized by lipopolysaccharide (LPS) into circulation. Black rice extract (BRE) has been known to have anti-inflammatory property. This study was conducted to investigate the effect of BRE on body weight (BW), waist circumference (WC), body mass index (BMI), ZO-1 and LPS of obese patients.

METHODS: Twenty-three male subjects were divided into non-obese group (NOG), obese group (COG) and BRE-obese group (BOG). Subjects in BOG received a daily dose of 5.6 g/day BRE for 4 weeks. BW, WC and BMI, serum ZO-1 and LPS were measured before and after treatment.

RESULTS: BRE was prepared successfully and free from microbial contamination. Treatment of BRE for 4 weeks reduce BW (95.40±5.78 vs. 94.59±6.00 kg, $p=0.043$), WC (109.25±3.55 vs. 107.50±3.46 cm, $p=0.0000$) BMI (32.65±1.86 vs. 32.18±1.80, $p=0.0000$) and LPS (222.27±38.63 vs. 131.63±9.70 ng/mL, $p=0.020$) of obese subjects. The pre-post ZO-1 levels in all groups were not significantly different ($p>0.05$).

CONCLUSION: Treatment of 5.6 gr BRE daily for four weeks can reduce BW, WC, BMI and serum LPS, but not serum ZO-1 in obese patients. Therefore, BRE may reduce inflammation in obesity.

KEYWORDS: black rice, obesity, BW, WC, BMI, LPS, ZO-1

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Introduction

Prevalence of obesity keep increasing rapidly, it was estimated that more than one billion people in the world are now living with obesity, nearly 880 million adults and 159 million children and adolescents aged 5-19 years, and about four million people die every year due to obesity and its comorbidities.(1-4) Obesity is an abnormal or excessive fat accumulation that may impair health due to an energy imbalance between calories consumed and calories expended.(5,6) Obesity is characterized by the increase of pro-inflammatory cytokines released from adipose tissue and the infiltration of leukocytes, especially macrophages, leading to chronic low-grade inflammation.(7,8)

Obesity has been associated with gut microbiota composition changes. One of the changes is an increase in Firmicutes and a decrease in Bacteroidetes, which will

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RESEARCH ARTICLE

Black Rice Extract Reduces Body Weight, Waist Circumference, Body Mass Index and Lipopolysaccharide in Obese Subjects: A Preliminary StudyArmanto Makmun^{1,*}, Agussalim Bukhari², Nurpudji Astuti Taslim², Aminuddin², Ferry Sandra^{3,4}¹Faculty of Medicine, Moslem Universitas Muslim Indonesia, Jl. Urip Sumoharjo Km.5, Makassar 90231, Indonesia²Department of Nutrition, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan Km.10, Makassar 90245, Indonesia³Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia⁴Center of Molecular Biology Study, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia

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excessive fat accumulation that may impair health due to an energy imbalance between calories consumed and calories expended.(5,6) Obesity is characterized by the increase of pro-inflammatory cytokines released from adipose tissue and the infiltration of leukocytes, especially macrophages, leading to chronic low-grade inflammation.(7,8)

Obesity has been associated with gut microbiota composition changes. One of the changes is an increase in Firmicutes and a decrease in Bacteroidetes, which will



contribute to the development of pro-inflammatory status in obesity through alteration in the intestinal barrier.(9) Zonula Occludens-1 (ZO-1) has been known to link tight junction proteins with the cytoskeleton and to provide integrity of the paracellular barrier, hence ZO-1 has been used as a biomarker of intestinal barrier integrity.(10,11) When the intestinal barrier was dysfunction, an endotoxin called lipopolysaccharide (LPS) could be transported into circulation. LPS has been reported to increased pro-inflammatory cytokines (12), therefore, the circulatory-transported LPS will cause metabolic endotoxemia and the production of pro-inflammatory cytokines leading to the development of chronic low-grade inflammation.(9)

Black rice is one variant of rice which has black pigment containing anthocyanins.(13) Compared with white rice, black rice has an abundance of phenolic compounds, which are associated with antioxidant activity. Black rice extract (BRE) was reported to have an anti-inflammatory effect on the splenocytes of a diabetes mellitus mouse model.(14) Another study also indicated that supplementation of BRE for 12 weeks had an effectiveness in reducing fat accumulation in postmenopausal women aged between 45 and 69 years.(15) Although the effects of BRE on oxidative stress and inflammation (16,17), hyperlipidaemia and hyperglycemia (18,19), body weight gain (20), lipid accumulation (21), and gut microbiota (22) have been elucidated, to our knowledge, the effect of BRE on intestinal barrier dysfunction and metabolic endotoxemia in subjects with obesity has not been clearly understood. Therefore, present study was conducted to investigate the effectiveness of BRE on ZO-1 and LPS in subjects with obesity.

Methods

Production of BRE Solution

From Toraja, South Sulawesi, 20 kg of Black rice (*Oryza sativa* L.) was obtained. The rice was milled into powder, macerated with 32 L of 70% ethanol, sonicated for 30 min, and left overnight. The next day, the solution was filtered, evaporated at 40°C, and dried at 60°C. Resulted paste was weighted, solubilized in sodium carboxymethylcellulose (Na-CMC), added with 0.5% citric acid to reach pH=3, and finally added with sorbitol to sweeten the solution.

Microbial Contamination Test

BRE solution was tested for possible contamination of microorganism with Total Plate Count (TPC) Analysis. Briefly, BRE was serial-diluted, poured and spread evenly

on Plate Count Agar (PCA), then incubated in an incubator at 37°C for 24 hours. After incubation, the formed colonies were counted.

For *Staphylococcus aureus* and *Salmonella* sp. tests, BRE solution was serial-diluted, spread evenly on Baird-Parker Agar (BPA) for *S. aureus* while Xylose Lysine Deoxycholate (XLD) Agar for *Salmonella* sp. Then the agar was incubated in an incubator at 37°C for 24 hours. After incubation, the formed colonies were counted.

Subject Recruitment and Criteria

Male subjects with age of 18-35 years old were recruited during the period of April-March 2021 at Hasanuddin University Medical Research Center (HUMRC) and at Ibnu Sina Hospital. Subjects with history of smoking, strict diet; chronic metabolic disorders (diabetes mellitus, hypertension, systemic lupus erythematosus, and rheumatoid arthritis) were excluded. Prior to the enrolment, all subject was informed and asked for their willingness to participate by signing a written informed consent form. This research protocol was approved by the Ethics Committee of the Faculty of Medicine, Hasanuddin University, Makassar (No. 300/UN4.6.4.5.31/PP36/2020). This study has been registered at clinicaltrials.gov under the registration number NCT04827628.

Anthropometric Measurement

Body weight (BW) was measured in kilogram (kg), body height was measured in centimetre (cm), waist circumference (WC) was measured in the half way between subjects' lowest rib and the top of the hipbone, Body Mass Index (BMI) was calculated as weight (kg) divided by height squared (m²). BMI score was used to differentiate between normal weight (18.5–22.9), overweight (23–24.9), or obesity (≥25).

Subject Intervention and Sample Collection

Subjects were divided into 3 groups: non-obese group (NOG), obese group (OG), and BRE-treated obese group (BOG) for 4 weeks. Serum ZO-1 and LPS was conducted before and after treatment with BRE. After overnight fasting, 5 mL venous blood was drawn, left at room temperature for 15 minutes, then centrifuged at 3000 rpm for 15 min. Afterward, the serum was collected, aliquoted and stored at –80°C for Enzyme-linked Immunosorbent Assay (ELISA) quantifications.

ELISA for ZO-1 and LPS

Collected serum was used to determine ZO-1 and LPS levels using Human Tight Junction Protein 1 (ZO-1) ELISA Kit

(Cat No. MBS2605490; MyBioSource, San Diego, CA, USA) and Human Lipopolysaccharides (LPS) ELISA Kit (Cat No. MBS266722; MyBioSource). Both kits utilized the double antibody sandwich ELISA technique. Anti-Human ZO-1 monoclonal antibody or anti-Human LPS monoclonal antibody was the precoated antibody, while a biotinylated polyclonal antibody was used as the detection antibody. The TMB that was used as the substrate, was reacted to form a blue product and finally turns to yellow after addition of the stop solution. For obtaining optical density (OD), microplate reader was set at 450nm. ZO-1 ELISA kit could detect at the range of 1.56-100 ng/mL with sensitivity of 0.5 ng/mL, while LPS ELISA kit could detect at the range of 15.6-1,000 ng/mL with sensitivity of 5 ng/mL.

Results

The BRE solution in concentration of 93.33 mg/mL and total volume of 28 L was prepared successfully. For the microbial test results, TPC for BRE was 4.6×10^3 CFU/g, while *S. aureus* and *Salmonella* sp. counts were both negative per 0.1 g of sample.

Forty male subjects were included in the study. Based on the BMI, 15 subjects were non-obese (included in NOG) and 25 subjects were obese. The obese subjects were then divided randomly into 2 groups: 12 subjects in OG and 13 subjects in BOG. Subjects in BOG consumed 60 mL BRE solution containing 5.6 g BRE daily for 4 weeks.

However, not all subjects could complete the study, 8 subjects in NOG, 4 subjects in OG and 5 subjects in BOG were dropped out due to their health conditions during the Coronavirus Disease 2019 (COVID-19) pandemics. Therefore, in the end of the of the study there were 7 subjects in NOG, 8 subjects in OG, and 8 subjects in BOG that completed the study and assessments. All subjects of all groups had similar age ($p=0.382$, Kruskal Wallis), for NOG 21.60 ± 0.61 years old, for OG 20.13 ± 0.91 years old and for BOG 22.33 ± 0.49 years old.

BRE reduced BW, WC and BMI

In the pre-treatment stage, subjects in OG and BOG had similar BW, WC and BMI, but higher than NOG. Treatment of BRE for 4 weeks could reduce significantly the BW, WC and BMI of obese subjects, as shown in the BOG (Table 1). The BW, WC and BMI of all groups were analysed further by calculating the pre-post differences (Δ) of each group (Figure 1). All Δ BW, Δ WC and Δ BMI showed significant differences between OG and BOG, suggesting that BRE could certainly reduce BW, WC and BMI of obese subjects.

BRE reduced LPS, but did not affect ZO-1

Similar to BW, WC and BMI, in the pre-treatment stage, subjects in OG and BOG had similar LPS level, but higher than NOG. Treatment of BRE for 4 weeks could also reduce significantly the LPS level of obese subjects, as shown in the BOG (Table 2). The ZO-1 level in NOG was higher than the one in OG and BOG. The pre-post ZO-1 levels in all groups were not significantly different.

Discussion

The current study showed that four weeks of BRE consumption can significantly reduce BW, WC, BMI and LPS, but not ZO-1 level. It has been widely reported that obesity is related with chronic inflammation, which is marked by LPS in the present study. The LPS are cellular wall components of gram-negative bacteria that contain a pathogen-associated molecular pattern, Lipid A, able to interact with the toll-like Receptor 4 via the myeloid differentiation primary response 88 protein. This interaction results in the activation of the pathway downstream and nuclear factor (NF)- κ B translocation, thus increasing the gene transcription of cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, and IL-6.(23) Normally, LPS concentrations are highest in the gut lumen and low or undetectable in the circulating plasma because LPS in the gut lumen do not penetrate the healthy intestinal epithelium.(24) BRE was known to contain high level

Table 1. Pre-post BW, WC and BMI of NOG, OG and BOG groups.

Parameter	NOG (n=7)			OG (n=8)			BOG (n=8)		
	Pre	Post	p-value	Pre	Post	p-value	Pre	Post	p-value
BW (kg)	60.39 \pm 3.04	60.33 \pm 3.03	0.103 [‡]	99.83 \pm 5.47	99.99 \pm 5.42	0.135 [‡]	95.40 \pm 5.78	94.59 \pm 6.00	0.043 ^{‡*}
WC (cm)	77.71 \pm 2.83	77.76 \pm 2.85	0.180 [#]	112.75 \pm 4.06	112.85 \pm 4.08	0.291 [#]	109.25 \pm 3.55	107.50 \pm 3.46	0.000 ^{‡*}
BMI	21.84 \pm 0.75	21.86 \pm 0.78	0.736 [‡]	34.08 \pm 1.58	33.96 \pm 1.65	0.831 [#]	32.65 \pm 1.86	32.18 \pm 1.80	0.000 ^{‡*}

Data are presented in mean \pm SEM. [‡]Paired-Samples T Test; [#]Wilcoxon Signed Rank Test; ^{*}significant with $p < 0.05$.

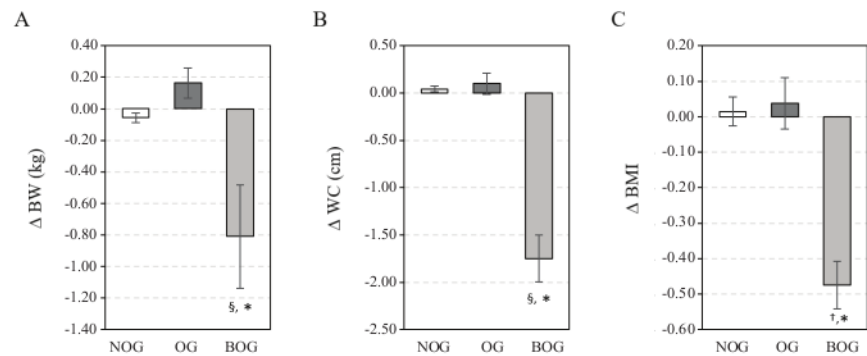


Figure 1. The Pre-post differences of BM, WC and BMI. Data of Table 1 was used to calculate the differences of pre-post of NOG, OG and BOG (mean±SEM). Δ: Pre-post difference. [§]Independent Samples T Test (compared with NOG); [†]Mann-Whitney Test (compared with NOG); *significant with $p<0.05$.

of anthocyanin. The anthocyanin in BRE, which has an anti-inflammatory effect, can modulate I-kappa-B-alpha (IκB-α) phosphorylation leading to lower expression of pro-inflammatory cytokines such as TNF-α, interferon (IFN)-γ, and ILs.(13)

It has been reported that pro-inflammatory cytokines regulated the tight junction protein ZO-1 expression.(10) Previous study reported also that high-fat diet feeding in mice could reduce the expression of ZO-1 in the jejunum. (25) In this study, the LPS level was reduced by BRE, however the ZO-1 level was not affected. Therefore, based on our present data, we suggested that the ZO-1 levels might not be detected well in the circulation. However, further larger cohort research is needed to clarify this issue.

providing material and non-material facilities, as well as infrastructure during the research. This research has been supported by grants from Faculty of Medicine, Universitas Muslim Indonesia.

Authors Contribution

AM and AB were involved in the conceptualization of the study, preparation of methodology, and the investigation. AM prepared the study resources and drafted the original manuscript. NAT and A gave critical suggestions. AM and FS performed the data analysis, prepared the visualization of the data, as well as revised and edited the manuscript,

Conclusion

Consumption of 5.6 gr BRE daily for four weeks can reduce BW, WC, BMI and serum LPS, but not serum ZO-1 in obese patients. Therefore, BRE may reduce inflammation in obesity.

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Table 2. Pre-post ZO-1 and LPS levels of NOG, OG and BOG groups.

Parameter	NOG (n=7)			OG (n=8)			BOG (n=8)		
	Pre	Post	p-value	Pre	Post	p-value	Pre	Post	p-value
ZO-1 (ng/mL)	17.26±2.09	17.55±2.97	0.932 [‡]	14.27±2.06	14.63±1.95	0.901 [‡]	14.62±2.77	14.41±1.25	0.954 [‡]
LPS (ng/mL)	149.00±20.83	139.82±14.35	0.778 [‡]	214.26±41.48	206.04±25.58	1.000 [#]	222.27±38.63	131.63±9.70	0.020 ^{†*}

Data are presented in mean±SEM. [‡]Paired-Samples T Test; [#]Wilcoxon Signed Rank Test; *significant with $p<0.05$.

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[InaBJ] M2024248 Editor Decision Round 1 - Resubmit for Review

Secretariat of InaBJ <secretariat@inabj.org>
To: armanto.makmun@umi.ac.id

Thu, Oct 10, 2024 at 10:46 AM

Dear Dr. Armanto Makmun,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "**Black Rice Extract Reduces Body Weight, Waist Circumference, Body Mass Index and Lipopolysaccharide in Obese Subjects: A Preliminary Study.**"

Our decision is to: **Resubmit for Review.**

This manuscript is interesting, however based on the peer-reviewers and editors review results, there are some issues that need to be revised. Please find the manuscript attached to see detailed comments.

Please make sure you read all the comments and revise the manuscript based on the suggestions given. Besides the comments our reviewers have given, please also pay attention to the use of English language, make sure you avoid any grammatical and diction errors.

Revise this manuscript thoroughly and according to the suggestions before **October 18, 2024**. Mark/highlighted the revised part of the manuscript, so that the editor will notice the changes. You are also obligated to provide a response letter with your response or the answer to reviewers' questions/comments. For an example on how to write a response letter, we also attach a response form template. Hopefully you find it well.

When you are done, you can upload it in: <https://inabj.org/index.php/ibj/author/submissionReview/3250>, or simply send us an email of your revised manuscript and response letter.

Please reply/notify us when you have received this email. If you have any questions, do not hesitate to contact us. Thank you for your attention. We wish you a nice day.

Best Regards,

--

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Black Rice Extract Reduces Body Weight, Waist Circumference, Body Mass Index and Lipopolysaccharide in Obese Subjects: A Preliminary Study

Abstract

Background: Leaky gut due to intestinal tight junction barrier dysfunction can induce inflammation in obesity. Zonula occludens-1 (ZO-1) plays a role in developing intestinal tight junction barrier dysfunction and gut microbiota imbalance, thus promote the translocation of bacterial endotoxin characterized by lipopolysaccharide (LPS) into circulation. Black rice extract (BRE) has been known to have anti-inflammatory property. This study was conducted to investigate the effect of BRE on body weight (BW), waist circumference (WC), body mass index (BMI), ZO-1 and LPS of obese patients.

Methods: Twenty-three male subjects were divided into non-obese group (NOG), obese group (COG) and BRE-obese group (BOG). Subjects in BOG received a daily dose of 5.6 g/day BRE for 4 weeks. BW, WC and BMI, serum ZO-1 and LPS were measured before and after treatment.

Results: BRE was prepared successfully and free from microbial contamination. Treatment of BRE for 4 weeks reduce BW, WC, BMI and LPS of obese subjects. The pre-post ZO-1 levels in all groups were not significantly different ($p>0.05$).

Conclusion: Consumption of 5.6 gr BRE daily for four weeks can reduce BW, WC, BMI and serum LPS, but not serum ZO-1 in obese patients. Therefore, BRE may reduce inflammation in obesity.

Keywords: black rice, obesity, BW, WC, BMI, LPS, ZO-1

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Commented [P12]: R1 #2: The abstract was initiated by the information which was not related to the purpose of the study which highlighted in the abstract. I suggest the authors to re-write or revise the Abstract section. There is also typo in the abstract, "conclusion" section.

Commented [P13]: R1 #3: The background section in the Abstract was no appropriate with the whole content and information in the manuscript. Therefore, I suggest major revision in this part.

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Introduction

Prevalence of obesity keep increasing rapidly, and it was estimated that about four million people die every year due to obesity and its comorbidities.^{A,B,C} Obesity is an abnormal or excessive fat accumulation that may impair health due to an energy imbalance between calories consumed and calories expended.^{1,D} ~~Obesity has been rapidly increased across the world every year.~~² Obesity is characterized by the increase of pro-inflammatory cytokines released from adipose tissue and the infiltration of leukocytes, especially macrophages, leading to chronic low-grade inflammation.^{3,E}

Obesity has been associated with gut microbiota composition changes. One of the changes is an increase in Firmicutes and a decrease in Bacteroidetes, which will contribute to the development of pro-inflammatory status in obesity through alteration in the intestinal barrier.⁴ Zonula Occludens-1 (ZO-1) has been known to link tight junction proteins with the cytoskeleton and to provide integrity of the paracellular barrier, hence ZO-1 has been used as a biomarker of intestinal barrier integrity.^{5,6} When the intestinal barrier was dysfunction, an endotoxin called Lipopolysaccharide (LPS) could be transported into circulation. LPS has been reported to increased pro-inflammatory cytokines^F, therefore, the circulatory-transported LPS will cause metabolic endotoxemia and the production of pro-inflammatory cytokines leading to the development of chronic low-grade inflammation.⁴

Black rice (*Oryza sativa* L.) is one variant of rice which has black pigment containing anthocyanins.⁷ Compared with white rice, black rice has an abundance of phenolic compounds, which are associated with antioxidant activity. Black rice extract (BRE) was reported to have an anti-inflammatory effect on the splenocytes of a diabetes mellitus mouse model.⁸ Another study also indicated that supplementation of BRE for 12 weeks had an effectiveness in reducing fat accumulation in postmenopausal women aged between 45 and 69 years.⁹ Although the effects of BRE on oxidative stress and inflammation^{10,11}, hyperlipidaemia and hyperglycemia^{12,13}, body

Commented [P15]: R2 #2: Please provide the prevalence number of obesity and its complication to highlight the importance of this topic to be studied.

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55 weight gain¹⁴, lipid accumulation¹⁵, and gut microbiota¹⁶ have been elucidated, to our knowledge,
56 the effect of BRE on intestinal barrier dysfunction and metabolic endotoxemia in subjects with
57 obesity has not been clearly understood. Therefore, present study was conducted to investigate
58 the effectiveness of BRE on ZO-1 and LPS in subjects with obesity.

59

60 **Methods**

61 **Production of BRE Solution**

62 From Toraja, South Sulawesi, 20 kg of Black rice (*Oryza sativa* L.) was obtained. The rice was
63 milled into powder, macerated with 32 L of 70% ethanol, sonicated for 30 min, and left overnight.

64 The next day, the solution was filtered, evaporated at 40°C, and dried at 60°C. Resulted paste
65 was weighted, solubilized in sodium carboxymethylcellulose (Na-CMC), added with 0.5% citric
66 acid to reach pH=3, and finally added with sorbitol to sweeten the solution.

67

68 **Microbial Contamination Test**

69 BRE solution was tested for possible contamination of microorganism with Total Plate Count
70 (TPC) Analysis. Briefly, BRE was serial-diluted, poured and spread evenly on Plate Count Agar
71 (PCA), then incubated in an incubator at 37°C for 24 hours. After incubation, the formed colonies
72 were counted.

73 For *Staphylococcus aureus* and *Salmonella sp.* tests, BRE solution was serial-diluted,
74 spread evenly on Baird-Parker Agar (BPA) for *Staphylococcus aureus* while Xylose Lysine
75 Deoxycholate (XLD) Agar for *Salmonella sp.* Then the agar was incubated in an incubator at
76 37°C for 24 hours. After incubation, the formed colonies were counted.

77

78 **Subject Recruitment and Criteria**

Commented [P17]: R1 #5: In the introduction section, authors wrote "...the effect of BRE on intestinal barrier dysfunction and metabolic endotoxemia in subjects with obesity has not been clearly understood. Therefore, present study was conducted to investigate the effectiveness of BRE on ZO-1 and LPS in subjects with obesity."

To my opinion, this is the highlight of the main purpose of this study. However, the study conducted and explain by the authors were not likely to be related to intestinal barrier dysfunction or metabolic endotoxemia. The pre-post BRE treatment of ZO-1 levels were found to be no significant different. Therefore, the purpose of study mentioned in the Introduction section should be revised.

Commented [P18]: R1 #6: How can the study purpose written in Line 9-10 was different to that written in Line 49-50? Please verify and write in a simple, but clear statement.

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79 Male subjects with age of 18-35 years old were recruited during the period of April-March 2021
80 at Hasanuddin University Medical Research Center (HUMRC) and at Ibnu Sina Hospital.
81 Subjects with history of smoking, strict diet; chronic metabolic disorders (diabetes mellitus,
82 hypertension, systemic lupus erythematosus, and rheumatoid arthritis) were excluded. Prior to
83 the enrolment, all subject was informed and asked for their willingness to participate by signing
84 a written informed consent form. This research protocol was approved by the Ethics Committee
85 of the Faculty of Medicine, Hasanuddin University, Makassar (No.
86 [300/UN4.6.4.5.31/PP36/2020](#)). This study has been registered at [clinicaltrials.gov](#) under the
87 registration number NCT04827628.

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88

89 **Anthropometric Measurement**

90 Body weight (BW) was measured in kilogram (Kg), body height was measured in centimetre
91 (cm), waist circumference (WC) was measured as..., Body Mass Index (BMI) was calculated as
92 weight (kg) divided by height squared (m^2). BMI score was used to differentiate between normal
93 weight (18.5–22.9), overweight (23–24.9), or obesity (≥ 25).

Commented [P19]: R2 #3: Please put in detail how the authors conducted WC measurement.

94

95 **Subject Intervention and Sample Collection**

96 ~~After BMI calculation,~~ Subjects were divided into 3 groups: non-obese group (NOG), obese
97 group (OG), and BRE-treated obese group (BOG) for 4 weeks. Serum ZO-1 and LPS was
98 conducted before and after treatment with BRE. After overnight fasting, 5 mL venous blood was
99 drawn, left at room temperature for 15 minutes, then centrifuged at 3000 rpm for 15 min.
100 Afterward, the serum was collected, aliquoted and stored at $-80^{\circ}C$ for Enzyme-linked
101 Immunosorbent Assay (ELISA) quantifications.

102

103 **ELISA for ZO-1 and LPS**

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105 Collected serum was used to determine ZO-1 and LPS levels using Human Tight Junction Protein
106 1 (ZO-1) ELISA Kit (Cat No. MBS2605490, MyBioSource, San diego, CA, USA) and Human
107 Lipopolysaccharides (LPS) ELISA Kit (Cat No. MBS266722, MyBioSource). Both kits utilized
108 double antibody sandwich ELISA technique. Anti-Human ZO-1 monoclonal antibody or anti-
109 Human LPS monoclonal antibody was the precoated antibody, while a biotinylated polyclonal
110 antibody was used as the detection antibody. TMB as the substrate, was reacted to form a blue
111 product and finally turns to yellow after addition of the stop solution. For obtaining optical
112 density (OD), microplate reader was set at 450nm. ZO-1 ELISA kit could detect at the range of
113 1.56-100 ng/mL with sensitivity of 0.5 ng/mL, while LPS ELISA kit could detect at the range of
114 15.6-1,000 ng/mL with sensitivity of 5 ng/mL.

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115

116 Results

Commented [PI10]: R1 #7: However, some of the interpretations was confusing.

117 BRE solution in concentration of 93.33 mg/mL and total volume of 28 L was prepared
118 successfully. For the microbial test results, TPC for BRE was 4.6×10^3 CFU/g, while
119 *Staphylococcus aureus* and *Salmonella sp.* counts were both negative per 0.1 g of sample.

120 Forty male subjects were included in the study. Based on BMI, 15 subjects were non-
121 obese (NOG) and 25 subjects were obese. The obese subjects were divided randomly into 2
122 groups: 12 subjects in OG and 13 subjects in BOG. Subjects in BOG consumed 60 mL BRE
123 solution containing 5.6 g BRE daily for 4 weeks. However, not all subjects could complete the
124 study, 8 subjects in NOG, 4 subjects in OG and 5 subjects in BOG were dropped out due to their
125 health conditions during the Coronavirus Disease 2019 (COVID-19) pandemics. Therefore, in
126 the end there were 7 subjects in NOG, 8 subjects in OG, and 8 subjects in BOG, completed the
127 study and assessments. All subjects of all groups had similar age ($p=...$), for NOG 21.60 ± 0.61
128 years old, for OG 20.13 ± 0.91 years old and for BOG 22.33 ± 0.49 years old.

Commented [PI11]: R2 #4: Please provide the significance of T-test for age.

129

131 **BRE reduces BW, WC and BMI**

132 In the pre-treatment stage, subjects in OG and BOG had similar body weight (BW), waist
 133 circumference (WC) and BMI, but higher than NOG. Treatment of BRE for 4 weeks could reduce
 134 significantly the BW, WC and BMI of obese subjects, as shown in the BOG (Table 1). The BW,
 135 WC and BMI of all groups were analysed further by calculating the pre-post differences (Δ) of
 136 each group (Figure 1). All Δ BW, Δ WC and Δ BMI showed significant differences between OG
 137 and BOG, suggesting that BRE could certainly reduce BW, WC and BMI of obese subjects.

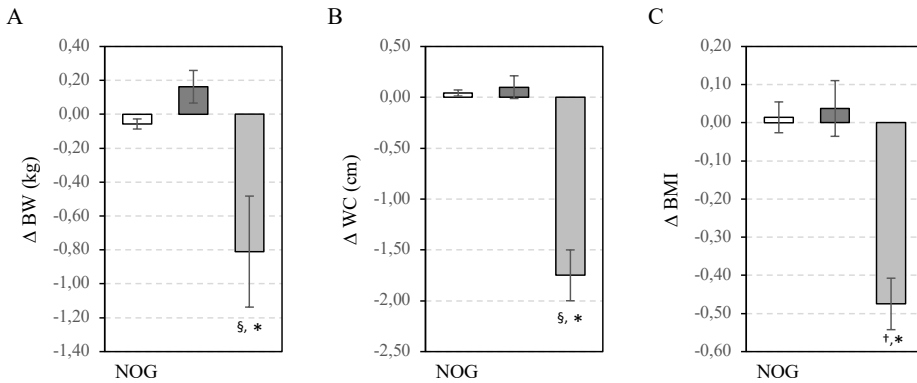
138

139 Table 1. Pre-post BM, WC and BMI of NOG, OG and BOG. (mean \pm SEM)

Parameter	NOG (n=7)			OG (n=8)			BOG (n=8)		
	Pre	Post	<i>p</i>	Pre	Post	<i>p</i>	Pre	Post	<i>p</i>
BW (kg)	60.39 \pm 3.04	60.33 \pm 3.03	0.103 [‡]	99.83 \pm 5.47	99.99 \pm 5.42	0.135 [‡]	95.40 \pm 5.78	94.59 \pm 6.00	0.043 ^{‡,*}
WC (cm)	77.71 \pm 2.83	77.76 \pm 2.85	0.180 [#]	112.75 \pm 4.06	112.85 \pm 4.08	0.291 [#]	109.25 \pm 3.55	107.50 \pm 3.46	0.000 ^{‡,*}
BMI	21.84 \pm 0.75	21.86 \pm 0.78	0.736 [‡]	34.08 \pm 1.58	33.96 \pm 1.65	0.831 [#]	32.65 \pm 1.86	32.18 \pm 1.80	0.000 ^{‡,*}

140 BW: Body weight; WC: Waist Circumference; BMI: Body Mass Index; NOG: Non-Obese Group; OG: Obese Group; BOG:
 141 BRE-treated Obese Group; [‡]Paired-Samples T Test; [#]Wilcoxon Signed Rank Test; **p*<0.05

142



143

144 Figure 1. Pre-post Differences of BM, WC and BMI. Data of Table 1 was used to calculate the
 145 differences of pre-post of NOG, OG and BOG (mean \pm SEM). Δ : Pre-post difference; BW: Body
 146 weight; WC: Waist Circumference; BMI: Body Mass Index; NOG: Non-Obese Group; OG:

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152 Obese Group; BOG: BRE-treated Obese Group; §Independent Samples T Test (compared with
 153 NOG); †Mann-Whitney Test (compared with NOG); * $p < 0.05$.
 154

155 **BRE reduced LPS, but did not affect ZO-1**

156 Similar to BW, WC and BMI, in the pre-treatment stage, subjects in OG and BOG had similar
 157 LPS level, but higher than NOG. Treatment of BRE for 4 weeks could also reduce significantly
 158 the LPS level of obese subjects, as shown in the BOG (Table 2). The ZO-1 level in NOG was
 159 higher than the one in OG and BOG. The pre-post ZO-1 levels in all groups were not significantly
 160 different.
 161

162 Table 2. Pre-post ZO-1 and LPS Levels of NOG, OG and BOG. (mean±SEM)

Parameter	NOG (n=7)			OG (n=8)			BOG (n=8)		
	Pre	Post	<i>p</i>	Pre	Post	<i>p</i>	Pre	Post	<i>p</i>
ZO-1 (ng/mL)	17.26±2.09	17.55±2.97	0.932 [‡]	14.27±2.06	14.63±1.95	0.901 [‡]	14.62±2.77	14.41±1.25	0.954 [‡]
LPS (ng/mL)	149.00±20.83	139.82±14.35	0.778 [‡]	214.26±41.48	206.04±25.58	1.000 [‡]	222.27±38.63	131.63±9.70	0.020 ^{‡,*}

163 ZO-1: Zonula Occludens-1; LPS: Lipopolysaccharide; NOG: Non-Obese Group; OG: Obese Group; BOG: BRE-treated Obese
 164 Group; ‡Paired-Samples T Test; #Wilcoxon Signed Rank Test; * $p < 0.05$
 165

166 **Discussion**

167 Our study showed that **four weeks of BRE consumption can significantly reduce BW,**
 168 **WC, BMI and LPS, but not ZO-1 level.** ↓

169 **LPS** are cellular wall components of gram-negative bacteria that contain a pathogen-
 170 associated molecular pattern, Lipid A, able to interact with the toll-like Receptor 4 via the
 171 myeloid differentiation primary response 88 protein. This interaction results in the activation of
 172 the pathway downstream and nuclear factor (NF)-κB translocation, thus increasing the gene
 173 transcription of cytokines such as TNF-α, IL-1, and IL-6.¹⁸ Normally, LPS concentrations are
 174 highest in the gut lumen and low or undetectable in the circulating plasma because LPS in the

Commented [PI12]: R2 #5: I believe all markers can be merged in one table so the readers can easily access all data. Please merge this with Table 1.

Commented [PI13]: R2 #6: Please start the discussion with a brief of study objective.

Commented [PI14]: R2 #7: Please find a possible explanation or mechanism on how treatment with BRE can reduce BW and WC, therefore the BMI.

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Commented [PI15]: R1 #8: The relation of LPS and obesity was explained in the background. However, the information provided was not clear enough and not supporting the Discussion section. Could author please revise?

178 gut lumen do not penetrate the healthy intestinal epithelium.¹⁹ BRE was known to contain high
179 level of anthocyanin. Anthocyanin in BRE, which has an anti-inflammatory effect, can modulate
180 I-kappa-B-alpha (IκB-α) phosphorylation leading to lower expression of pro-inflammatory
181 cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and
182 interleukins (ILs).¹⁸

183 It has been reported that pro-inflammatory cytokines regulated the tight junction protein
184 ZO-1 expression.[1] Previous study reported also that high-fat diet feeding in mice could reduce
185 the expression of ZO-1 in jejunum.¹⁷ In our study, the LPS level was reduced by BRE, however
186 the ZO-1 level was not affected. Therefore, based on our present data, we suggested that the ZO-
187 1 levels might not be detected well in the circulation. However, further larger cohort research is
188 needed to clarify this issue.

189

190 Conclusion

191 Consumption of 5.6 gr BRE daily for four weeks can reduce BW, WC, BMI and serum LPS, but
192 not serum ZO-1 in obese patients. Therefore, BRE may reduce inflammation in obesity.

193

194 References

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Commented [P116]: R2 #8: Please elaborate more to explain how ZO level is not significantly different instead of different LPS level in this study, rather than just "technical issue".

Commented [P117]: R2 #9: Is there any studies exploring the safety and efficacy of consuming BRE in prolonged time? The authors may add the information here.

What is the authors' suggestion based on this study for future perspective?

Commented [P118]: R2 #10: Please recheck the reference number in the text, since there will be some modifications.

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[InaBJ] M2024248 Editor Decision Round 1 - Resubmit for Review

Armanto Makmun <armanto.makmun@umi.ac.id>
To: Secretariat of InaBJ <secretariatinabj@gmail.com>

Mon, Oct 14, 2024 at 6:57 PM

dear Secretariat of The Indonesian Biomedical Journal;

Here we send you our revised manuscript.

We are very happy to be able to work with InaBJ.

Thank you a lot.

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2 attachments

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Black Rice Extract Reduces Body Weight, Waist Circumference, Body Mass Index and Lipopolysaccharide in Obese Subjects: A Preliminary Study

Abstract

Background: The prevalence of obesity, or an excessive fat accumulation, is keep increasing. In obesity, inflammation can be induced by leaky gut due to the intestinal tight junction barrier dysfunction. Zonula occludens-1 (ZO-1) plays a role in developing intestinal tight junction barrier dysfunction and gut microbiota imbalance, thus promote the translocation of bacterial endotoxin characterized by lipopolysaccharide (LPS) into circulation. Black rice extract (BRE) has been known to have anti-inflammatory property. This study was conducted to investigate the effect of BRE on body weight (BW), waist circumference (WC), body mass index (BMI), ZO-1 and LPS of obese patients.

Methods: Twenty-three male subjects were divided into non-obese group (NOG), obese group (COG) and BRE-obese group (BOG). Subjects in BOG received a daily dose of 5.6 g/day BRE for 4 weeks. BW, WC and BMI, serum ZO-1 and LPS were measured before and after treatment.

Results: BRE was prepared successfully and free from microbial contamination. Treatment of BRE for 4 weeks reduce BW (95.40 ± 5.78 vs. 94.59 ± 6.00 kg, $p=0.043$), WC (109.25 ± 3.55 vs. 107.50 ± 3.46 cm, $p=0.000$) BMI (32.65 ± 1.86 vs. 32.18 ± 1.80 , $p=0.000$) and LPS (222.27 ± 38.63 vs. 131.63 ± 9.70 ng/mL, $p=0.020$) of obese subjects. The pre-post ZO-1 levels in all groups were not significantly different ($p>0.05$).

Conclusion: Treatment of 5.6 gr BRE daily for four weeks can reduce BW, WC, BMI and serum LPS, but not serum ZO-1 in obese patients. Therefore, BRE may reduce inflammation in obesity.

Keywords: black rice, obesity, BW, WC, BMI, LPS, ZO-1

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Commented [P12]: R1 #2: The abstract was initiated by the information which was not related to the purpose of the study which highlighted in the abstract. I suggest the authors to re-write or revise the Abstract section. There is also typo in the abstract, "conclusion" section.

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Introduction

Prevalence of obesity keep increasing rapidly, it was estimated that more than one billion people in the world are now living with obesity, nearly 880 million adults and 159 million children and adolescents aged 5-19 years, and about four million people die every year due to obesity and its comorbidities.¹⁻⁴ Obesity is an abnormal or excessive fat accumulation that may impair health due to an energy imbalance between calories consumed and calories expended.^{5,6} Obesity is characterized by the increase of pro-inflammatory cytokines released from adipose tissue and the infiltration of leukocytes, especially macrophages, leading to chronic low-grade inflammation.^{7,8}

Obesity has been associated with gut microbiota composition changes. One of the changes is an increase in Firmicutes and a decrease in Bacteroidetes, which will contribute to the development of pro-inflammatory status in obesity through alteration in the intestinal barrier.⁹ Zonula Occludens-1 (ZO-1) has been known to link tight junction proteins with the cytoskeleton and to provide integrity of the paracellular barrier, hence ZO-1 has been used as a biomarker of intestinal barrier integrity.^{10,11} When the intestinal barrier was dysfunction, an endotoxin called Lipopolysaccharide (LPS) could be transported into circulation. LPS has been reported to increased pro-inflammatory cytokines¹², therefore, the circulatory-transported LPS will cause metabolic endotoxemia and the production of pro-inflammatory cytokines leading to the development of chronic low-grade inflammation.⁹

Black rice (*Oryza sativa* L.) is one variant of rice which has black pigment containing anthocyanins.¹³ Compared with white rice, black rice has an abundance of phenolic compounds, which are associated with antioxidant activity. Black rice extract (BRE) was reported to have an anti-inflammatory effect on the splenocytes of a diabetes mellitus mouse model.¹⁴ Another study also indicated that supplementation of BRE for 12 weeks had an effectiveness in reducing fat accumulation in postmenopausal women aged between 45 and 69 years.¹⁵ Although the effects of BRE on oxidative stress and inflammation^{16,17}, hyperlipidaemia and hyperglycemia^{18,19}, body

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51 weight gain²⁰, lipid accumulation²¹, and gut microbiota²² have been elucidated, to our knowledge,
52 the effect of BRE on intestinal barrier dysfunction and metabolic endotoxemia in subjects with
53 obesity has not been clearly understood. Therefore, present study was conducted to investigate
54 the effectiveness of BRE on ZO-1 and LPS in subjects with obesity.

55

56 **Methods**

57 **Production of BRE Solution**

58 From Toraja, South Sulawesi, 20 kg of Black rice (*Oryza sativa* L.) was obtained. The rice was
59 milled into powder, macerated with 32 L of 70% ethanol, sonicated for 30 min, and left overnight.

60 The next day, the solution was filtered, evaporated at 40°C, and dried at 60°C. Resulted paste
61 was weighted, solubilized in sodium carboxymethylcellulose (Na-CMC), added with 0.5% citric
62 acid to reach pH=3, and finally added with sorbitol to sweeten the solution.

63

64 **Microbial Contamination Test**

65 BRE solution was tested for possible contamination of microorganism with Total Plate Count
66 (TPC) Analysis. Briefly, BRE was serial-diluted, poured and spread evenly on Plate Count Agar
67 (PCA), then incubated in an incubator at 37°C for 24 hours. After incubation, the formed colonies
68 were counted.

69 For *Staphylococcus aureus* and *Salmonella sp.* tests, BRE solution was serial-diluted,
70 spread evenly on Baird-Parker Agar (BPA) for *Staphylococcus aureus* while Xylose Lysine
71 Deoxycholate (XLD) Agar for *Salmonella sp.* Then the agar was incubated in an incubator at
72 37°C for 24 hours. After incubation, the formed colonies were counted.

73

74 **Subject Recruitment and Criteria**

Commented [P17]: R1 #5: In the introduction section, authors wrote "...the effect of BRE on intestinal barrier dysfunction and metabolic endotoxemia in subjects with obesity has not been clearly understood. Therefore, present study was conducted to investigate the effectiveness of BRE on ZO-1 and LPS in subjects with obesity."

To my opinion, this is the highlight of the main purpose of this study. However, the study conducted and explain by the authors were not likely to be related to intestinal barrier dysfunction or metabolic endotoxemia. The pre-post BRE treatment of ZO-1 levels were found to be no significant different. Therefore, the purpose of study mentioned in the Introduction section should be revised.

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75 Male subjects with age of 18-35 years old were recruited during the period of April-March 2021
76 at Hasanuddin University Medical Research Center (HUMRC) and at Ibnu Sina Hospital.
77 Subjects with history of smoking, strict diet; chronic metabolic disorders (diabetes mellitus,
78 hypertension, systemic lupus erythematosus, and rheumatoid arthritis) were excluded. Prior to
79 the enrolment, all subject was informed and asked for their willingness to participate by signing
80 a written informed consent form. This research protocol was approved by the Ethics Committee
81 of the Faculty of Medicine, Hasanuddin University, Makassar (No.
82 300/UN4.6.4.5.31/PP36/2020). This study has been registered at *clinicaltrials.gov* under the
83 registration number NCT04827628.

84

85 **Anthropometric Measurement**

86 Body weight (BW) was measured in kilogram (Kg), body height was measured in centimetre
87 (cm), waist circumference (WC) was measured in the halfway between subjects' lowest rib and
88 the top of the hipbone, Body Mass Index (BMI) was calculated as weight (kg) divided by height
89 squared (m^2). BMI score was used to differentiate between normal weight (18.5–22.9),
90 overweight (23–24.9), or obesity (≥ 25).

91

92 **Subject Intervention and Sample Collection**

93 Subjects were divided into 3 groups: non-obese group (NOG), obese group (OG), and BRE-
94 treated obese group (BOG) for 4 weeks. Serum ZO-1 and LPS was conducted before and after
95 treatment with BRE. After overnight fasting, 5 mL venous blood was drawn, left at room
96 temperature for 15 minutes, then centrifuged at 3000 rpm for 15 min. Afterward, the serum was
97 collected, aliquoted and stored at $-80^{\circ}C$ for Enzyme-linked Immunosorbent Assay (ELISA)
98 quantifications.

99

Commented [PI9]: R2 #3: Please put in detail how the authors conducted WC measurement.

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100 ELISA for ZO-1 and LPS

101 Collected serum was used to determine ZO-1 and LPS levels using Human Tight Junction Protein
102 1 (ZO-1) ELISA Kit (Cat No. MBS2605490, MyBioSource, San diego, CA, USA) and Human
103 Lipopolysaccharides (LPS) ELISA Kit (Cat No. MBS266722, MyBioSource). Both kits utilized
104 double antibody sandwich ELISA technique. Anti-Human ZO-1 monoclonal antibody or anti-
105 Human LPS monoclonal antibody was the precoated antibody, while a biotinylated polyclonal
106 antibody was used as the detection antibody. TMB as the substrate, was reacted to form a blue
107 product and finally turns to yellow after addition of the stop solution. For obtaining optical
108 density (OD), microplate reader was set at 450nm. ZO-1 ELISA kit could detect at the range of
109 1.56-100 ng/mL with sensitivity of 0.5 ng/mL, while LPS ELISA kit could detect at the range of
110 15.6-1,000 ng/mL with sensitivity of 5 ng/mL.

111

112 Results

113 BRE solution in concentration of 93.33 mg/mL and total volume of 28 L was prepared
114 successfully. For the microbial test results, TPC for BRE was 4.6×10^3 CFU/g, while
115 *Staphylococcus aureus* and *Salmonella sp.* counts were both negative per 0.1 g of sample.

116 Forty male subjects were included in the study. Based on BMI, 15 subjects were non-
117 obese (NOG) and 25 subjects were obese. The obese subjects were divided randomly into 2
118 groups: 12 subjects in OG and 13 subjects in BOG. Subjects in BOG consumed 60 mL BRE
119 solution containing 5.6 g BRE daily for 4 weeks. However, not all subjects could complete the
120 study, 8 subjects in NOG, 4 subjects in OG and 5 subjects in BOG were dropped out due to their
121 health conditions during the Coronavirus Disease 2019 (COVID-19) pandemics. Therefore, in
122 the end there were 7 subjects in NOG, 8 subjects in OG, and 8 subjects in BOG, completed the
123 study and assessments. All subjects of all groups had similar age ($p=0.382$, Kruskal Wallis), for
124 NOG 21.60 ± 0.61 years old, for OG 20.13 ± 0.91 years old and for BOG 22.33 ± 0.49 years old.

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125

126 **BRE reduces BW, WC and BMI**

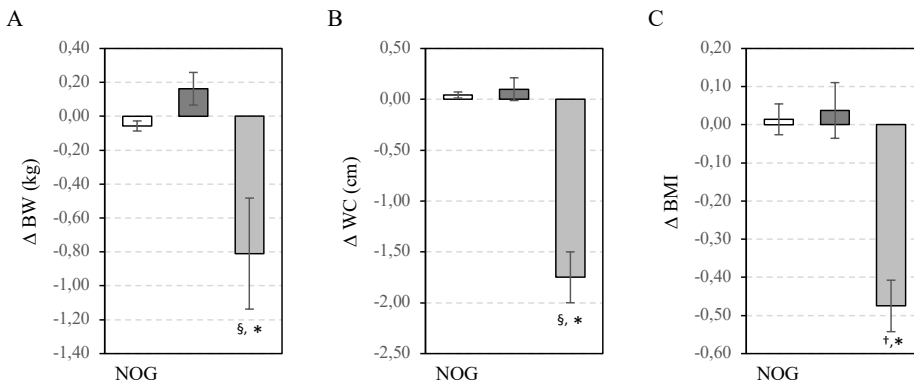
127 In the pre-treatment stage, subjects in OG and BOG had similar body weight (BW), waist
 128 circumference (WC) and BMI, but higher than NOG. Treatment of BRE for 4 weeks could reduce
 129 significantly the BW, WC and BMI of obese subjects, as shown in the BOG (Table 1). The BW,
 130 WC and BMI of all groups were analysed further by calculating the pre-post differences (Δ) of
 131 each group (Figure 1). All Δ BW, Δ WC and Δ BMI showed significant differences between OG
 132 and BOG, suggesting that BRE could certainly reduce BW, WC and BMI of obese subjects.

133

134 Table 1. Pre-post BM, WC and BMI of NOG, OG and BOG. (mean \pm SEM)

Parameter	NOG (n=7)			OG (n=8)			BOG (n=8)		
	Pre	Post	<i>p</i>	Pre	Post	<i>p</i>	Pre	Post	<i>p</i>
BW (kg)	60.39 \pm 3.04	60.33 \pm 3.03	0.103 [‡]	99.83 \pm 5.47	99.99 \pm 5.42	0.135 [‡]	95.40 \pm 5.78	94.59 \pm 6.00	0.043 ^{‡,*}
WC (cm)	77.71 \pm 2.83	77.76 \pm 2.85	0.180 [#]	112.75 \pm 4.06	112.85 \pm 4.08	0.291 [#]	109.25 \pm 3.55	107.50 \pm 3.46	0.000 ^{‡,*}
BMI	21.84 \pm 0.75	21.86 \pm 0.78	0.736 [‡]	34.08 \pm 1.58	33.96 \pm 1.65	0.831 [#]	32.65 \pm 1.86	32.18 \pm 1.80	0.000 ^{‡,*}

135 BW: Body weight; WC: Waist Circumference; BMI: Body Mass Index; NOG: Non-Obese Group; OG: Obese Group; BOG:
 136 BRE-treated Obese Group; [‡]Paired-Samples T Test; [#]Wilcoxon Signed Rank Test; **p*<0.05
 137



138

139 Figure 1. Pre-post Differences of BM, WC and BMI. Data of Table 1 was used to calculate the
 140 differences of pre-post of NOG, OG and BOG (mean \pm SEM). Δ : Pre-post difference; BW: Body
 141 weight; WC: Waist Circumference; BMI: Body Mass Index; NOG: Non-Obese Group; OG:

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142 Obese Group; BOG: BRE-treated Obese Group; §Independent Samples T Test (compared with
 143 NOG); †Mann-Whitney Test (compared with NOG); * $p < 0.05$.
 144

145 **BRE reduced LPS, but did not affect ZO-1**

146 Similar to BW, WC and BMI, in the pre-treatment stage, subjects in OG and BOG had similar
 147 LPS level, but higher than NOG. Treatment of BRE for 4 weeks could also reduce significantly
 148 the LPS level of obese subjects, as shown in the BOG (Table 2). The ZO-1 level in NOG was
 149 higher than the one in OG and BOG. The pre-post ZO-1 levels in all groups were not significantly
 150 different.

151

152 Table 2. Pre-post ZO-1 and LPS Levels of NOG, OG and BOG. (mean±SEM)

Parameter	NOG (n=7)			OG (n=8)			BOG (n=8)		
	Pre	Post	<i>p</i>	Pre	Post	<i>p</i>	Pre	Post	<i>p</i>
ZO-1 (ng/mL)	17.26±2.09	17.55±2.97	0.932 [‡]	14.27±2.06	14.63±1.95	0.901 [‡]	14.62±2.77	14.41±1.25	0.954 [‡]
LPS (ng/mL)	149.00±20.83	139.82±14.35	0.778 [‡]	214.26±41.48	206.04±25.58	1.000 [‡]	222.27±38.63	131.63±9.70	0.020 ^{‡,*}

153 ZO-1: Zonula Occludens-1; LPS: Lipopolysaccharide; NOG: Non-Obese Group; OG: Obese Group; BOG: BRE-treated Obese
 154 Group; ‡Paired-Samples T Test; #Wilcoxon Signed Rank Test; * $p < 0.05$
 155

156 **Discussion**

157 Our study showed that four weeks of BRE consumption can significantly reduce BW,
 158 WC, BMI and LPS, but not ZO-1 level. It has been widely reported that obesity is related with
 159 chronic inflammation, which is marked by LPS in the present study. The LPS are cellular wall
 160 components of gram-negative bacteria that contain a pathogen-associated molecular pattern,
 161 Lipid A, able to interact with the toll-like Receptor 4 via the myeloid differentiation primary
 162 response 88 protein. This interaction results in the activation of the pathway downstream and
 163 nuclear factor (NF)-κB translocation, thus increasing the gene transcription of cytokines such as
 164 TNF-α, IL-1, and IL-6.²³ Normally, LPS concentrations are highest in the gut lumen and low or

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Commented [P115]: R1 #8: The relation of LPS and obesity was explained in the background. However, the information provided was not clear enough and not supporting the Discussion section. Could author please revise?

165 undetectable in the circulating plasma because LPS in the gut lumen do not penetrate the healthy
166 intestinal epithelium.²⁴ BRE was known to contain high level of anthocyanin. Anthocyanin in
167 BRE, which has an anti-inflammatory effect, can modulate I-kappa-B-alpha (IκB-α)
168 phosphorylation leading to lower expression of pro-inflammatory cytokines such as tumor
169 necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukins (ILs).¹³

170 It has been reported that pro-inflammatory cytokines regulated the tight junction protein
171 ZO-1 expression.¹⁰ Previous study reported also that high-fat diet feeding in mice could reduce
172 the expression of ZO-1 in jejunum.²⁵ In our study, the LPS level was reduced by BRE, however
173 the ZO-1 level was not affected. Therefore, based on our present data, we suggested that the ZO-
174 1 levels might not be detected well in the circulation. However, further larger cohort research is
175 needed to clarify this issue.

177 Conclusion

178 Consumption of 5.6 gr BRE daily for four weeks can reduce BW, WC, BMI and serum LPS, but
179 not serum ZO-1 in obese patients. Therefore, BRE may reduce inflammation in obesity.

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Commented [PI16]: R2 #8: Please elaborate more to explain how ZO level is not significantly different instead of different LPS level in this study, rather than just "technical issue".

Commented [PI17]: R2 #9: Is there any studies exploring the safety and efficacy of consuming BRE in prolonged time? The authors may add the information here.

What is the authors' suggestion based on this study for future perspective?

Commented [PI18]: R2 #10: Please recheck the reference number in the text, since there will be some modifications.

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266

R1 #4: The information in this part was not supporting the data of this study. Therefore, I suggest revising this section, especially the information explaining the importance of ZO-1.

Response: Thanks for your comment. Information about gut microbiota was written, due to the correlation of inflammation with obesity. We realized that investigation on microbiota was not carried out in this study, but we think that the microbiota information could be a complementary information as one of the factors associated with inflammation in obesity.

R2 #5: I believe all markers can be merged in one table so the readers can easily access all data. Please merge this with Table 1.

Response: Thanks for your suggestion. However, we would like to show the readers the anthropometric measurements in Table 1, then in Table 2, we proposed to show the biomarker results for tight-junction and inflammation. Therefore, we separated the table into Table 1 and Table 2.

R2 #6: Please start the discussion with a brief of study objective.

Response: Thanks for your suggestion. As we know this could be the writing preference among the authors. In our case, we prefer to start the Discussion with our major finding, which is the most important part to be discussed.

R2 #7: Please find a possible explanation or mechanism on how treatment with BRE can reduce BW and WC, therefore the BMI.

Response: Thanks for your question. During the study, most of the subjects reported that they were experiencing better gastrointestinal movement. Prior to the study, most of the subjects were constipated. Therefore, this phenomenon might cause reduction of BW, WC and BMI. However, this should be investigated further to be certain, hence, we did not include this explanation in the Discussion.

R1 #8: The relation of LPS and obesity was explained in the background. However, the information provided was not clear enough and not supporting the Discussion section. Could author please revise?

Response: Thanks for your suggestion. A sentence was added to link obesity to chronic inflammatory, leading to LPS, as the marker in this study.

R2 #8: Please elaborate more to explain how ZO level is not significantly different instead of different LPS level in this study, rather than just “technical issue”.

Response: Thanks for your comment. When we designed this study, we expected that the BRE could increase the ZO-1 expression. As we know that the ZO-1 expression is related with tight-junction, so when the tight-junction was increased/improved, the LPS release-to-circulation could be inhibited. However, in the present study the ZO-1 expression was not improved significantly, therefore should be investigated further in larger number of subjects.

R2 #9: Is there any studies exploring the safety and efficacy of consuming BRE in prolonged time? The authors may add the information here.

Response: Thanks for your question. In the present time, we couldn't find any report on the safety and efficacy of consuming BRE in the prolonged time. We could just speculate that the BRE might be consumed for a long period, since black rice (not the extract) has been regularly consumed by local people in Makassar since long ago. However, this should be also be investigated since the raw material could be different from its extract.

R2 #10: What is the authors' suggestion based on this study for future perspective?

Response: Thanks for your question. This research should be investigated further to disclose the component of BRE, to investigate the possible effect of BRE on the microbiota, to investigate the safety and efficacy of BRE consumption in the prolonged time, to recheck the ZO-1 expression by using different method/kit/sample collection, and hopefully in the future the BRE could be consumed as a healthy drink.

[InaBJ] M2024248 Editor Decision - Manuscript Accepted

Secretariat of InaBJ <secretariatinabj@gmail.com>
To: Armanto Makmun <armanto.makmun@umi.ac.id>
Cc: Ferry Sandra <ferry@trisakti.ac.id>

Tue, Oct 22, 2024 at 4:29 PM

Dear Dr. Armanto Makmun,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "**Black Rice Extract Reduces Body Weight, Waist Circumference, Body Mass Index and Lipopolysaccharide in Obese Subjects: A Preliminary Study.**"

Our decision is to: **Accept Manuscript.**

Congratulations on your interesting research, and thank you for allowing us to publish this valuable material. Please let us know once you have read this email. We wish you a nice day.

Best Regards,

--

Secretariat of The Indonesian Biomedical Journal

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Certificate for Author M2024248 - Armanto Makmun [signed].pdf

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C E R T I F I C A T E O F A C K N O W L E D G M E N T

N o : 1 5 0 / C . 0 1 / I B J / 2 0 2 4

The board of Indonesian Biomedical Journal awarding this certificate to:

Armanto Makmun, Agussalim Bukhari, Ferry Sandra

as recognition of an ACCEPTED paper entitled

**“Black Rice Extract Reduces Body Weight,
Waist Circumference, Body Mass Index and
Lipopolysaccharide in Obese Subjects: A Preliminary Study”**

that will be published in The Indonesian Biomedical Journal.

Jakarta, October 16, 2024

Editor in Chief

The Indonesian
Biomedical Journal

Dr. Dewi Muliaty

SURAT TUGAS

NOMOR : 579/BKD/FKG-USAKTI/X/2024

Dekan Fakultas Kedokteran Gigi Universitas Trisakti

Dasar : Sehubungan dengan kegiatan publikasi penelitian dan penulisan Jurnal Ilmiah yang dilaksanakan oleh Para Dosen/Staf Pengajar Fakultas Kedokteran Gigi Universitas Trisakti.

MENUGASKAN :

Kepada : drg. Ferry Sandra, Ph.D

Untuk : Melakukan penulisan pada jurnal ilmiah dengan judul : *Black Rice Extract Reduces Body Weight, Waist Circumference, Body Mass Index and Lipopolysaccharide in Obese Subjects: A Preliminary Study* yang dipublikasikan di bulan Oktober 2024 pada The Indonesian Biomedical Journal.

Demikian agar tugas tersebut dilaksanakan dengan penuh rasa tanggung jawab.

Ditetapkan di : Jakarta

Pada tanggal : 1 Oktober 2024

Dekan,



drg. Wiwiek Poedjiastoeti, M.Kes., Sp.BMM., Ph.D.