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Content

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REVIEW ARTICLE

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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=0.000) 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

Indones Biomed J. 2024; 16(5): 459-63

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 (m^2). 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 x 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 ΔBW, ΔWC and ΔBMI 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)-κB 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±3.04	60.33±3.03	0.103‡	99.83±5.47	99.99±5.42	0.135‡	95.40±5.78	94.59±6.00	0.043‡*
WC (cm)	77.71±2.83	77.76±2.85	0.180#	112.75±4.06	112.85±4.08	0.291#	109.25±3.55	107.50±3.46	0.000‡*
BMI	21.84±0.75	21.86±0.78	0.736‡	34.08±1.58	33.96±1.65	0.831#	32.65±1.86	32.18±1.80	0.000‡*

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

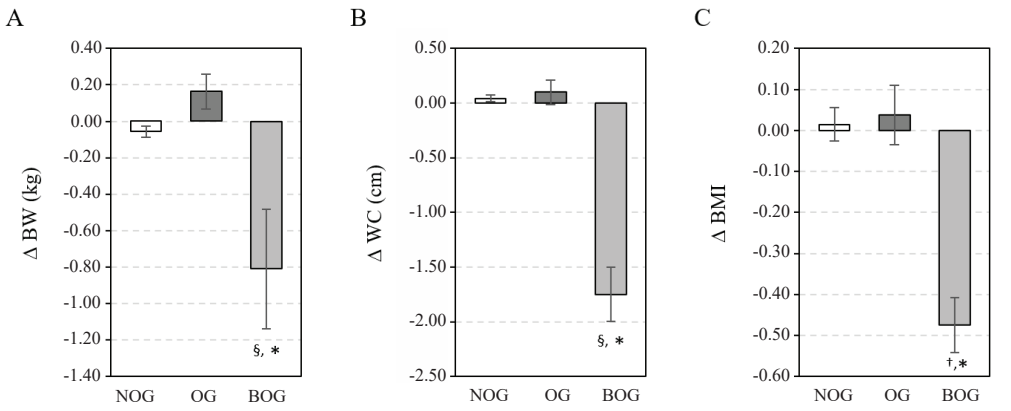

Figure 1. The Pre-post differences of BM, WC and BMI. Data of Table 1 was used to calculate the differences of pre-post of NOG, OG and BOG (mean±SEM). Δ: Pre-post difference. §Independent Samples T Test (compared with NOG); †Mann-Whitney Test (compared with NOG); *significant with $p<0.05$.

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.

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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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,

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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

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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

Indones Biomed J. 2024; 16(5): 459-63

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 (m^2). 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 x 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 ΔBW, ΔWC and ΔBMI 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)-κB 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±3.04	60.33±3.03	0.103‡	99.83±5.47	99.99±5.42	0.135‡	95.40±5.78	94.59±6.00	0.043‡*
WC (cm)	77.71±2.83	77.76±2.85	0.180#	112.75±4.06	112.85±4.08	0.291#	109.25±3.55	107.50±3.46	0.000‡*
BMI	21.84±0.75	21.86±0.78	0.736‡	34.08±1.58	33.96±1.65	0.831#	32.65±1.86	32.18±1.80	0.000‡*

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

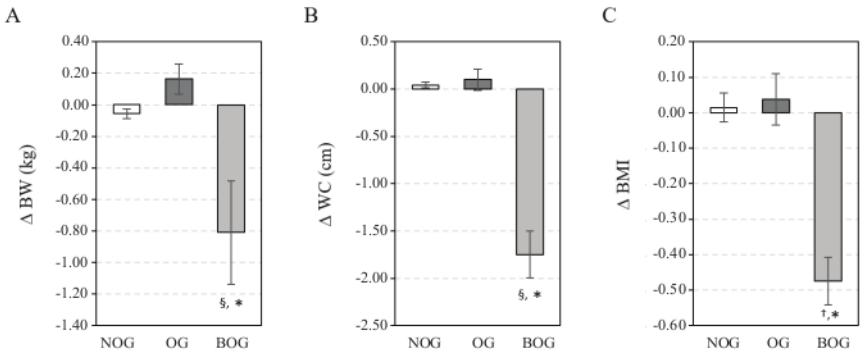

Figure 1. The Pre-post differences of BM, WC and BMI. Data of Table 1 was used to calculate the differences of pre-post of NOG, OG and BOG (mean±SEM). Δ: Pre-post difference. §Independent Samples T Test (compared with NOG); †Mann-Whitney Test (compared with NOG); *significant with $p<0.05$.

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.

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.

Acknowledgments

The author expresses his deepest gratitude to the Hasanuddin University and Moslem University of Indonesia for 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,

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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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Secretariat of InaBJ <secretariatinabj@gmail.com> Thu, Oct 10, 2024 at 10:46 AM
To: armanto.makmun@umi.ac.id

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.

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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 ($p>0.05$).

Conlusion: 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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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

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weight gain[14], lipid accumulation[15], and gut microbiota[16] 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 *Staphylococcus 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

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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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Anthropometric Measurement

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

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Subject Intervention and Sample Collection

~~After BMI calculation,~~ 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

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Forty male subjects were included in the study. Based on BMI, 15 subjects were non-obese (NOG) and 25 subjects were obese. The obese subjects were 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 there were 7 subjects in NOG, 8 subjects in OG, and 8 subjects in BOG, completed the study and assessments. All subjects of all groups had similar age (p=…), 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.

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BRE reduces BW, WC and BMI

In the pre-treatment stage, subjects in OG and BOG had similar body weight (BW), waist circumference (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 ΔBW, ΔWC and ΔBMI showed significant differences between OG and BOG, suggesting that BRE could certainly reduce BW, WC and BMI of obese subjects.

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

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

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

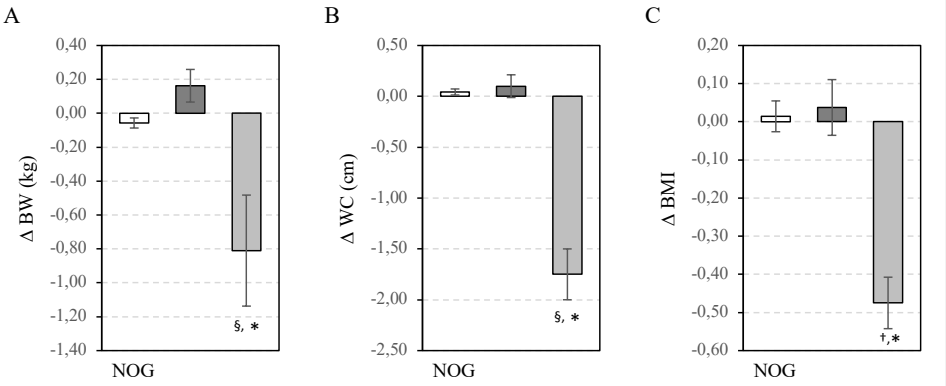

Figure 1. Pre-post Differences of BM, WC and BMI. Data of Table 1 was used to calculate the differences of pre-post of NOG, OG and BOG (mean±SEM). Δ: Pre-post difference; BW: Body weight; WC: Waist Circumference; BMI: Body Mass Indes; NOG: Non-Obese Group; OG:

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

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.

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	*p*	Pre	Post	*p*	Pre	Post	*p*
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‡,*

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

Discussion

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

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)-κB translocation, thus increasing the gene transcription of cytokines such as TNF-α, IL-1, and IL-6.[18] Normally, LPS concentrations are highest in the gut lumen and low or undetectable in the circulating plasma because LPS in the

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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?

gut lumen do not penetrate the healthy intestinal epithelium.[19] BRE was known to contain high level of anthocyanin. 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 tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukins (ILs).[18]

It has been reported that pro-inflammatory cytokines regulated the tight junction protein ZO-1 expression.[1] Previous study reported also that high-fat diet feeding in mice could reduce the expression of ZO-1 in jejunum.[17] In our 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.

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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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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Armanto Makmun <armanto.makmun@umi.ac.id> Mon, Oct 14, 2024 at 6:57 PM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>

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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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 [PI2]: 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 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 (*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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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 *Staphylococcus 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.

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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 (m^2). BMI score was used to differentiate between normal weight (18.5–22.9), overweight (23–24.9), or obesity (≥25).

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ELISA for ZO-1 and LPS

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Results

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 x 10^3 CFU/g, while *Staphylococcus aureus* and *Salmonella sp*. counts were both negative per 0.1 g of sample.

Forty male subjects were included in the study. Based on BMI, 15 subjects were non-obese (NOG) and 25 subjects were obese. The obese subjects were 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 there were 7 subjects in NOG, 8 subjects in OG, and 8 subjects in BOG, 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.

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

BRE reduces BW, WC and BMI

In the pre-treatment stage, subjects in OG and BOG had similar body weight (BW), waist circumference (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 ΔBW, ΔWC and ΔBMI showed significant differences between OG and BOG, suggesting that BRE could certainly reduce BW, WC and BMI of obese subjects.

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

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

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

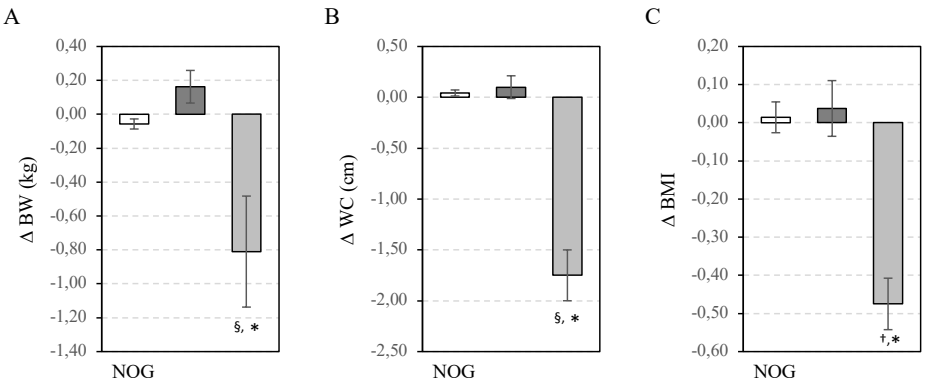

Figure 1. Pre-post Differences of BM, WC and BMI. Data of Table 1 was used to calculate the differences of pre-post of NOG, OG and BOG (mean±SEM). Δ: Pre-post difference; BW: Body weight; WC: Waist Circumference; BMI: Body Mass Indes; NOG: Non-Obese Group; OG:

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

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.

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**	***p***	**Pre**	**Post**	***p***	**Pre**	**Post**	***p***
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‡,*

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

Discussion

Our 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)-κB translocation, thus increasing the gene transcription of cytokines such as 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 [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.

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?

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 of anthocyanin. 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 tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukins (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 jejunum.[25] In our 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.

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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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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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, leasing 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.

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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**."

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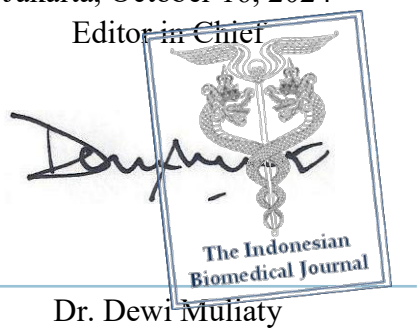

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Demikian agar tugas tersebut dilaksanakan dengan penuh rasa tanggung jawab.

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Pada tanggal : 1 Oktober 2024

Dekan,

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