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Zonulin as a Responsive Marker for Lifestyle Intervention: A Preliminary Study

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Abstract Insulin Resistance (IR) is the onset pathological condition that emerges before the occurrence of type 2 diabetes mellitus (T2DM). The development of IR condition is very difficult to observe. The initial stage of this condition does not show significant symptoms. Therefore, biomarkers are needed to detect this condition early and accurately. Zonulin is a protein that plays a role in regulating intestinal permeability and might be potential as an IR biomarker. Recent studies have shown the association of zonulin with T2DM and other metabolic diseases. However, there have been no studies showing zonulin profile in subjects at risk of IR and its response towards healthy lifestyle intervention. Therefore, this study aimed to determine the profile of zonulin in the blood plasma of subjects at risk of IR (n = 16 persons) relative to T2DM subjects (n = 3 persons) and healthy subjects (n = 42 persons), as well as changes after the intervention of red rice consumption and physical exercise for 3 months. The intervention included red rice consumption five days per week (250 grams / serving), physical exercise 150 minutes per week, and counseling once every two weeks. Plasma zonulin was measured semi-quantitatively using the sandwich ELISA method. The results showed a significant difference in plasma zonulin OD ($p < 0.001$) between the three groups. In T2DM and IR subjects, plasma zonulin profile was 9.9 times (2.437 ± 0.290) and 3.1 times (0.767 ± 0.575) higher than healthy subjects (0.246 ± 0.167) respectively. After 3 months of red rice consumption and physical exercise, there was no significant change in glycemic profile, in both IR and healthy subject. Interestingly, the plasma zonulin in IR subjects decreased significantly ($p = 0.019$), to an average value (0.276 ± 0.106) which was almost the same as the healthy subjects (0.264 ± 0.170). Thus, zonulin appeared to be more responsive as a marker of lifestyle intervention than the commonly used glycemic parameters.

Keywords Zonulin, Insulin Resistance, Diabetes Mellitus, Lifestyle Intervention, Early Marker

1. Introduction

Diabetes mellitus is the 9th biggest cause of death in the world. The number of people with diabetes mellitus has increased fourfold over the past 30 years. One in eleven adults are predicted to have diabetes mellitus. The epidemic of diabetes mellitus is observed to be the highest in Asia [1]. Indonesia has the 7th highest number of people with diabetes mellitus in the world. People with diabetes mellitus in Indonesia are estimated at 10 million in 2015 [2]. Of the total population of people suffering diabetes mellitus, an estimated 87-91% have type 2 diabetes mellitus (T2DM) [3].

Insulin resistance (IR) is the initial pathological condition that precedes type 2 diabetes mellitus (T2DM). The development of IR condition is very difficult to observe because it does not show significant symptoms [4]. Symptoms usually indicate that the patient has entered the chronic stage of T2DM and becomes more difficult to treat. Therefore, a biomarker is needed to detect this disease early and accurately.

Zonulin is a regulatory protein that plays a role in increasing gut intestinal permeability. The presence of zonulin is able to regulate tight junctions, create oxidative stress, and trigger an inflammatory response [5]. Several studies have shown the potential of zonulin as a biomarker for health status related to various metabolic diseases. High zonulin profile in the blood was observed in obese [6], T2DM [7], and non-alcoholic fatty liver disease subjects [8] compared to the healthy group. Thus, zonulin might be potential as a biomarker of IR condition.

Unhealthy eating habits, such as high sugar and fat consumption, and sedentary lifestyles are the risk factors for developing T2DM. Although genetic factors also play a role in increasing the risk of T2DM, unhealthy lifestyles are important factors that determine the etiology of this disease. The risk of T2DM could be reduced through a healthy diet, maintaining ideal body weight, regular daily exercise, and avoiding smoking and alcohol [9,10]. However there have

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been no studies showing zonulin profiles in subjects at risk of IR; and their response to healthy lifestyle intervention.

In this study, an analysis of plasma zonulin profile in subjects at risk of IR was conducted, relative to T2DM and healthy subjects. Lifestyle intervention such as consumption of red rice and exercise was also conducted to determine the changes in the plasma zonulin profile in IR and healthy subjects. This study was expected to provide an initial description of the plasma zonulin profile in subjects at risk of IR, and to assess the response of zonulin as biomarker for lifestyle changes to prevent further pathogenesis of IR condition.

2. Materials and Methods

Study participants were Indonesian urban citizens selected from employees of PT Nutrifood Indonesia. A total of 61 participants consisting of 27 men and 34 women were involved in this study. This research design consisted of cross-sectional and intervention study. Intervention stage was carried out at Nutrifood Research Center, PT Nutrifood Indonesia, Jl. Rawabali II No. 3 Jakarta which lasted from September to December 2017. Meanwhile, measurement of plasma zonulin was carried out at the Laboratory of Animal Structure and Development at School of Life Science and Technology - Bandung Institute of Technology, from January to June 2018. This study was approved by the Atma Jaya Catholic University's Research Ethics Commission with ethics number FR-UAJ-26-13 / R0.

2.1. Research Design

2.1.1. Cross-sectional Study

This study was conducted to compare the plasma zonulin profile in subjects at risk of IR with HOMA-IR greater than 1.55 [11] (n = 16 persons, 11 males), T2DM subjects with fasting plasma glucose greater than 125 mg / dL [12] (n = 3 persons, all males), and healthy subjects with HOMA-IR values less than or equal to 1.55 (n = 42 persons, 13 males). The inclusion criteria were adults aged 25-50 years and no smoking habits. The exclusion criteria were pregnant and breastfeeding women, and the consumption of prescription medicine.

2.1.2. Intervention Study

This study was conducted for 12-weeks using subjects at risk of IR (n = 9 persons, 6 males) and healthy subjects (n = 8 persons, 3 males). At week 0, participants were measured based on the anthropometric parameters, blood biochemical parameters, total calorie, and nutritional intake (through 3 x 24 hour food recall). Anthropometric parameters include the body fat and skeletal muscle mass were measured using the InBody 720 device. The measurement of the blood biochemical parameters include fasting plasma glucose and fasting plasma insulin were performed by commercial laboratory. Participants were also asked to complete the International Physical Activity Questionnaire (IPAQ). The intervention included recommending red rice consumption five days per week (250 grams / serving) [13], sports activity 150 minutes per week [14], and counseling once every two weeks [15]. Nutritional intake and IPAQ were re-measured in the 6th and 12th week to assure all participants followed the intervention guideline. Blood biochemical and anthropometry parameters were re-measured in the 12th week.

2.2. Plasma Zonulin Measurement

The plasma zonulin concentration was measured using the human zonulin ELISA kit (MyBiosource), and was performed on plasma samples obtained at week 0 and week 12. The measurement was repeated twice.

Zonulin specific antibodies had been coated on microplates. Blood plasma samples were added as much as 100 μl into each well, then closed using adhesive strips. The microplates were then incubated at 37°C for 2 hours. The liquid from each well was discarded. Biotin-conjugated antibodies (1x) were added as much as 100μl into each well and incubated for 1 hour at 37°C. Then the liquid in the well was removed and rinsed with a wash buffer (200 μl) and allowed to stand for 2 minutes then discarded. This rinsing procedure was repeated 2 times. One hundred μl of horseradish peroxidase (HRP) that was conjugated with avidin (1x) was added to each well and covered with adhesive strip. Then, the samples were incubated at 37°C for 1 hour. The rinsing procedure was carried again for 5 more times. The 90 μl tetramethylbenzidine (TMB) substrate was added to each well and incubated for 15 minutes at 37°C (avoided from light). A total of 50 μl stop solution was added to each well. The sample was read using a microplate reader at a wavelength of 450 nm within 5 minutes.

2.3. Statistic Analysis

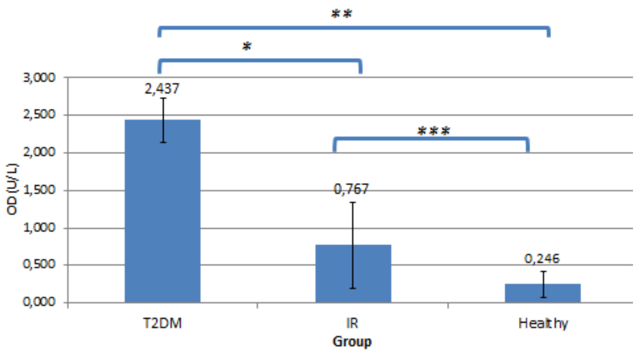
The results were analyzed using the SPSS Statistics version 25 application (IBM, Armonk, New York, United States). In the cross-sectional study, data analysis was conducted using the Kruskal-Wallis test and Mann Whitney U test, while the results of the intervention study were analyzed using paired T-test. Secondary data which includes anthropometric parameters and blood biochemistry parameters was processed using a paired T-test or Wilcoxon signed-rank test to determine differences in profiles before and after intervention.

3. Results and Discussion

3.1. Plasma Zonulin Profile in Subjects at Risk of Insulin Resistance, Type 2 Diabetes Mellitus, and Healthy Subjects

The presence of zonulin in blood plasma was analyzed semi-quantitatively using the sandwich ELISA method. The

ELISA method provides an output in the form of optical density (OD) values. Analysis of plasma zonulin profiles was conducted in 16 persons at risk of IR, 3 persons of type 2 diabetes mellitus (T2DM), and 42 healthy persons. The optical density of plasma zonulin profile of the three groups is shown in **Figure 1**.



*p=0,007; **p=0,004; ***p<0,001

Figure 1. Plasma zonulin profiles of T2DM, IR, and healthy subjects

The ELISA results showed that the plasma zonulin OD profile in IR subjects (0.767 ± 0.575) tended to be lower than T2DM subjects (*p=0.007*) and higher than healthy subjects (*p<0.001*). The highest OD was observed in T2DM subjects (2.437 ± 0.290), which was 9.9 times higher than healthy subjects (0.246 ± 0.167). This was consistent with the research by Zhang et al. (2014) [7], which stated that serum zonulin profiles of T2DM patients were observed to be higher in T2DM patients than in healthy groups.

Plasma zonulin profile of the IR group (0.767 ± 0.576) tended to be 3.1 times higher than the healthy group (0.246 ± 0.167), although the standard deviation of plasma zonulin in the IR group was quite high. This was thought to be related to the pathogenesis of IR towards T2DM which involves impaired pancreatic beta cell function. The pathogenesis stages consist of compensation stages (increase in insulin secretion), beta cell adaptation, transient unstable period, stable decompensation, and severe decompensation [16]. The high variation in OD values in the IR group was thought to be influenced by different stages of pathogenesis in IR subjects.

The high plasma zonulin profile in the blood can be assumed to be associated with the increased intestinal permeability [17]. In this study, plasma zonulin profile was found to be related to the intestinal zonulin profile. The presence of zonulin in the intestines of IR group was thought to be related to an increase in the intestinal permeability. This was evidenced by Sapone et al. (2006) [18] which showed a positive correlation between serum zonulin and intestinal permeability which was validated by lactulose/mannitol test in humans. Research conducted by Ohlsson et al. (2017) [19] also showed a positive correlation between zonulin in faeces and zonulin in blood serum in T2DM subjects.

In this study, the higher plasma zonulin profile in the IR group was also thought to contribute to an inflammatory response that plays a role in the pathogenesis of T2DM. Antigen molecules can more easily enter the bloodstream by diffusion with an increased intestinal permeability which results in a low grade inflammatory response [20]. This low grade inflamation was also characterized by an increase of TNFα and IL-6 proinflammatory cytokines in the T2DM group compared to healthy group [6,7].

3.2. Blood Biochemical Profile, Anthropometric Parameters, and Plasma Zonulin Profile after the Intervention of Red Rice Consumption and Exercise

IR condition could be prevented by a healthy lifestyle before becoming a severe condition of diabetes mellitus. Healthy food consumption accompanied by exercise could be a solution for IR condition. Meanwhile, diabetes mellitus need further medical treatment. We would like to see the changes of blood biochemical profile, anthropometric parameters, and plasma zonulin profile after lifestyle intervention in IR and healthy subjects. Changes in blood biochemical profiles after the intervention of red rice consumption and physical exercise can be seen in **Table 1** (for IR subjects) and **Table 2** (for healthy subjects).

Table 1. Blood Biochemical Profile of IR Subjects

Parameter	Before	After	Δ	*p*
FPG (mg/dL)	88.44 ± 9.07	88.11 ± 11.88	-0.33 ± 7.04	0.890
FPI (mU/L)	9.20 ± 2.97	9.03 ± 4.70	-0.17 ± 2.51	0.843
HOMA-IR	2.06 ± 0.96	2.08 ± 1.50	0.02 ± 0.71	0.964

Table 2. Blood Biochemical Profile of IR Subjects

Parameter	Before	After	Δ	*p*
FPG (mg/dL)	75.13 ± 3.40	78.63 ± 5.10	3.5 ± 4.47	0.062
FPI (mU/L)	5.64 ± 1.48	6.61 ± 6.04	0.96 ± 5.63	0.674
HOMA-IR	1.05 ± 0.30	1.32 ± 1.30	0.27 ± 1.20	0.944

FPG = fasting plasma glucose; FPI = fasting plasma insulin; HOMA-IR = homeostasis model assessment for insulin resistance

Blood biochemical profile including FPG and FPI, as well as HOMA-IR showed no significant changes after the intervention in both IR and healthy subject after 3 months of red rice consumption and physical exercise. Although there was no significant change in blood biochemical profile, the intervention of red rice consumption and physical exercise showed significant changes in anthropometric parameters, especially in IR subjects. The profile of the IR subjects' and healthy subjects' anthropometric parameters before and after the intervention can be seen in **Table 3 and Table 4**, respectively.

The parameters related to body fat (FM, VFA, and PBF) decreased significantly in IR subjects; yet, there was no significant change in healthy subjects. The intervention did reduce body fat parameters that correlate with IR risk, similar to the research conducted by Kosaka et al., (2005) [21]. However, the intervention did not significantly increase skeletal muscle mass. The 150 minutes recommendation belonged to the aerobic exercise, which was intended in fat burning, meanwhile muscle building is usually mediated by resistance training. Nonetheless, the intervention succeeded in reducing body fat parameters and was thought to contribute in lowering the risk of IR.

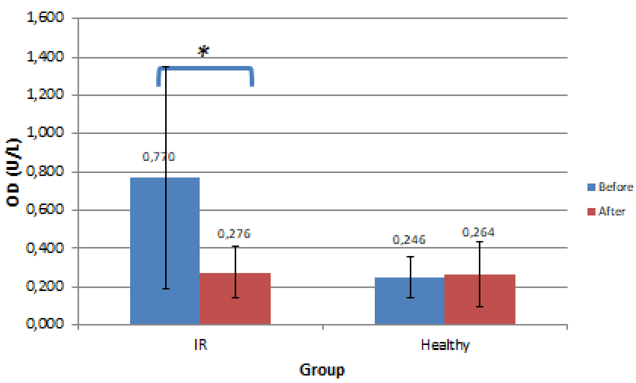
Table 3. Anthropometric Parameters of IR Subjects

Parameter	Before	After	Δ	*p*
BM (kg)	78.51±13.65	76.88±13.96	-1.63±2.32	0.068
FM (kg)	25.72±4.74	24.09±4.35	-1.63±2.08	0.046*
VFA (cm^2)	101.11±15.73	95.28±16.79	-5.83±6.43	0.026*
BMI (kg/m^2)	27.10±3.45	26.60±3.35	-0.50±0.71	0.068
PBF (%)	33.01±5.06	31.47±3.72	-1.54±1.99	0.048*
WHR	0.92±0.04	0.91±0.05	-0.01±0.02	0.188
SMM (kg)	29.53±6.89	29.57±6.69	0.04±0.56	0.819

Table 4. Anthropometric Parameters of Healthy Subjects

Parameter	Before	After	Δ	*p*
BM (kg)	68.94±10.66	66.55±9.13	-2.39±3.80	0.119
FM (kg)	23.28±6.80	21.05±6.00	-2.23±2.94	0.070
VFA (cm^2)	86.05±20.74	78.03±19.61	-8.03±11.27	0.084
BMI (kg/m^2)	26.10±3.20	25.25±2.85	-0.85±1.33	0.114
PBF (%)	33.90±8.47	31.95±8.65	-1.95±2.98	0.107
WHR	0.88±0.04	0.87±0.04	-0.02±0.03	0.109
SMM (kg)	25.28±6.08	26.34±6.12	1.06±3.09	0.363

BM= body mass; FM= fat mass; VFA= visceral fat area; BMI= body mass index; PBF= body fat percentage; SMM= skeletal muscle mass; WHR= waist to hip ratio



**p=0,019*

Figure 2. Plasma Zonulin Profile Before and After Intervention

This study was also intended to examine the ability of zonulin as a biomarker of the healthy lifestyle intervention in IR subjects. Despite there was no significant change in the glycemic responses such as FPG, FPI, and HOMA IR; our study showed a significant change in plasma zonulin profiles in IR subjects, but not in the healthy subjects, after the intervention period. Decreased plasma zonulin was aligned with the improvement of some anthropometric parameters (FM, VFA, PBF). Plasma zonulin profile before and after intervention can be seen in **Figure 2**.

The results showed a significant decrease in plasma zonulin of IR subjects (*p=0.019*). In healthy subjects there was a slight increase in plasma zonulin, but the value was not statistically significant (*p=0.669*). After the intervention, we observed a decrease in the plasma zonulin OD profile in IR subjects (0.276 ± 0.132), to be similar to that of healthy subjects (0.264 ± 0.168). This was thought to indicate a similar intestinal permeability profile between healthy subjects and IR subjects after intervention. Spearman correlation test results showed a significant positive correlation between plasma zonulin with BM *(r=0.646; p=0.005*), SMM (*r=0.515; p=0.035*), and HOMA-IR (*r=0.542; p=0.024*).

Intervention of red rice consumption and exercise succeeded in reducing blood plasma zonulin. Other blood biochemical parameters (FPG, FPI, and HOMA-IR) did not show significant changes. This result indicated that zonulin was relatively more sensitive than other biochemical parameters related to changes in healthy lifestyles. Moreover, zonulin profile also decreased along with the improvement of some anthropometric parameters (FM, VFA, and PBF). Zonulin appeared to have the potential to be an IR biomarker and healthy lifestyle intervention assessment. More validation of measurements with higher number of samples is needed to obtain strong conclusions that support the role of zonulin related to IR pathogenesis.

4. Conclusions

This study indicated that the profile of plasma zonulin in subjects at risk of IR was observed to be 3,2 times lower than in T2DM subjects and 3,1 times higher than in healthy subjects. To our knowledge, this was the first study to investigate the zonulin profile in IR subjects, since the previous studies focused in healthy, T2DM, or other digestive disorder. Furthermore, zonulin appeared to be more responsive towards lifestyle intervention compared to commonly used glycemic parameters. Thus, zonulin has the potential to be explored as an early marker in area of diabetics study.

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