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Research Article

Hypoglycemic Effect of Analog Rice Made from Modified Cassava Flour (Mocaf), Arrowroot Flour and Kidney Bean Flour on STZ-NA Induced Diabetic Rats

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Abstract

Background and Objective: Analog rice made from modified cassava flour (mocaf), arrowroot and kidney beans contains high level of dietary fiber and resistant starch potentially consumed as functional food, particularly for diabetes mellitus. However, its hypoglycemic property has not been comprehensively investigated. After previous research on analog rice made from mocaf and kidney beans flour as protein source, arrowroot flour was added to the formulation due to its hypoglycemic effect. The aim of the study was to evaluate hypoglycemic effect of analog rice made from mocaf, arrow root and kidney beans on Streptozotocin-Nicotinamide (STZ-NA) induced diabetic rats. **Materials and Methods:** Twenty four male Wistar rats were randomly divided into 4 groups, 6 rats each, healthy rats fed with standard feed (H) and three diabetic groups, respectively fed with standard feed (DM), rice variety C4 and mocaf, arrowroot and kidney beans based analog rice (AR). **Results:** During 4 weeks intervention, feed consumption, body weight and blood glucose level were measured once a week. The results indicated that diabetic rats fed with analog rice (AR) had the highest blood glucose level reduction (55.07%), significantly higher than C4 (18.91%). Total Short Chain Fatty Acids (SCFA) concentration of groups fed with analog rice and rice was 53.96 and 50.76 mmol L⁻¹, respectively. AR group also had higher Langerhans islets of 10.60 than C4 group of 6.80. The analog rice treatment decreased the blood glucose level. **Conclusion:** The results indicated that RS and dietary fiber was responsible to glucose reduction effect by analog rice diet through SCFA as resistant starch fermentation product in colon.

Key words: Analog rice, blood glucose, short chain fatty acids, Langerhans islets cells, diabetes mellitus

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rice is the main carbohydrate source for Indonesians as it provides more than 60% of energy needs. However, as high rice consumption might exacerbate diabetes, it is increasingly avoided by diabetes mellitus. Diabetes mellitus is chronic metabolic disease characterized by blood glucose level (hyperglycemia) induced by insulin deficiency or resistance¹. More than 90% of starch consuming humans suffer from type 2 diabetes mellitus² which due to insulin resistance. It is estimated that 4% of world population are living with diabetes and might increase to 5.4% by 2025³, projected to rise to 8.8% in 2015 and 10.4% by the year 2040 among 20-79 years old adults⁴.

Continuous medication over the years by those with diabetes prompts concern of undesired side effect, starchent boredom and high cost. Thus, diet management through hypoglycemic food consumption might help to improve their diabetic condition. Resistant starch (RS) and dietary fiber rich diet is reportedly able to improve glucose response of those with diabetes^{5,6}. As resistant starch cannot be hydrolyzed by amylase and difficult to digest in the small intestine⁷, it moves to colon and transformed into Short Chain Fatty Acids (SCFA) through fermentation by microflora. Several factors determine resistant starch amount, such as amylose and amylopectin ratio, cooking and the presence of other substances which prevent the enzyme's contact with starch⁸. Previous researches reported on efficacy of high resistant starch products to reduce blood glucose, both in animal model⁹⁻¹⁴ and human¹⁵⁻¹⁹. Resistant starch was also reported to be able to improve insulin response²⁰⁻²².

Analog rice made from mocaf (fermented cassava flour), arrowroot and kidney beans was reportedly rich of resistant starch. A research by Wahjuningsih and Haslina²³ revealed that analog rice (artificial or non-paddy rice with carbohydrate content similar or higher than those of rice, usually made from combination of flour from local commodities) made from mocaf, arrowroot and kidney beans (50:40:10) contain dietary fiber and resistant starch of 9.15 and 10.49%, respectively. Hence this research aimed to confirm its hypoglycemic property on STZ-NA induced diabetic rats.

MATERIALS AND METHODS

Mocaf, arrowroot flour and kidney beans flour were the main material. Healthy 2-3 months old male white Wistar rats weighed 200-250 g obtained from Animal Experiment Unit, Integrated Research and Testing Laboratory of Universitas Gadjah Mada, Indonesia. Other materials were rice variety C4, GOD-PAP reagent, 0.1 M sodium citrate buffer (pH 4.5), 10%

glucose solution, streptozotocin, nicotinamide, ELISA kit for insulin (Elabscience Biotechnology Co., Ltd), 90% ethanol and standard feed consist of casein, mineral mix, vitamin mix, L-cysteine, Choline bitartrate, corn starch, soybean oil and dietary fiber.

Preparation of products

Mocaf preparation: Cassava was peeled, cleanly washed, sliced at 2 mm thick and fermented for 3 days. Fermented cassava was dried at 50°C for 12 h and ground into mocaf.

Arrowroot flour preparation: Arrowroot was peeled, cleanly washed, sliced at 2 mm thick and blanched at 70°C for 5 min. After drained, arrowroot was dried at 50°C for 12 h.

Kidney beans flour preparation: Kidney beans flour was made according to procedure previously used by Wahjuningsih and Kunarto²⁴. Kidney beans was soaked for 12 h, washed and blanched at 70°C for 20 min before dried at 50°C for 12 h.

Analog rice preparation: Analog rice was prepared from mocaf, arrowroot flour and kidney beans flour, added with glyceryl monostearate, carrageenan, coconut oil and salt previously dissolved in water half of total ingredient. After mixed for 5 min, mixture was steamed for 15 min then put into single thread extruder EXT-D01SC. Mixture was stirred again for 2 min then molded into analog rice. Formed granules were then dried in cabinet dryer at 50°C for 12 h²⁵.

Animal testing methods: The study was approved by the Experimental Animal Unit, Center of Food and Nutrition Studies, Universitas Gadjah Mada, Indonesia in April-June, 2017. Wistar rats were individually caged in conditioned rooms (28-32 °C, 50-60% relative humidity). Water and diet were available *ad libitum* during experiments. Twenty four Wistar rats were randomly divided into 4 groups, 6 rats each. Group 1 was healthy rats (H) fed with AIN93M standard feed²⁶. Group 2, 3 and 4 were diabetic induced rat, respectively fed with standard diet (DM), rice variety C4 and analog rice (AR). Rats were intraperitoneally given using nicotinamide (NA) dissolved in 0.9% NaCl buffer at dosage of 230 mg kg⁻¹, 15 min before induction using streptozotocin (STZ) at dosage of 60 mg kg⁻¹ body weight²⁷. To prevent mortality due to hypoglycemic effect, 5% glucose solution was given in drinking water during 24 h after induction²⁸. Five days after induction, glucose level measurement was conducted on blood sample taken from

Table 1: Composition of experiment diets (g kg⁻¹)

Composition (g)	AIN 93M	C4	AR
Corn starch	620	-	-
Rice	-	834	-
Analog rice	-	-	873
Casein	140	82	93
Sucrose	100	100	100
Soybean oil	40	35	27
Dietary fiber	50	50	6.4
Mineral mix	35	22	13.2
Vitamin mix	10	10	10
L-cysteine	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5

retro orbital vein using microcapillary method. Rats were classified as diabetic at minimum fasting glucose level of 200 mg dL⁻¹. Intervention feed was given for 4 weeks. Once a week feed intake, body weight and blood glucose level were analyzed. Iso-protein and iso-calory AIN93M feed formula²⁶ is presented in Table 1. Experiments condition was permitted by Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Universitas Gadjah Mada, Indonesia, number 414/KEC-LPPT/XII/2015.

Parameters assessed

Feed intake and body weight measurement: Feed intake was measured daily by remaining feed weighing. Body weight was weighed once a week.

Blood glucose level analysis: Fasting glucose analysis was conducted using Glucose Oxidase Phenol Aminophenazone (GOD-PAP) method²⁹ on blood taken from retro orbital vein using microcapillary method.

Short Chain Fatty Acids (SCFA) analysis: At the end of the study, rats were anesthetized and euthanized, digesta in secum was taken to measure SCFA level, consist of acetate, propionate and butyrate using gas chromatography. Digesta was weighed and centrifuged at 10000 rpm for 15 min. After supernatant separation, sample was directly injected into GCMS column (Shimadzu GC 8A, with FID detector).

Langerhans islets cells analysis: To measure Langerhans islets level, hematoxylin eosin staining method was applied³⁰. Initially, paraffin was removed from pancreas tissue through deparaffination using xylol. Tissue was placed into xylol III for 3 min, to xylol II for 3 min and xylol I for 5 min. The next step was rehydration to restore fluid to the tissue using alcohol solution. During first rehydration, tissue was soaked into absolute alcohol III, II, I for 3 min each, then into 95, 90, 80% alcohol for 3 min each and into 70% alcohol for 5 min. Tissue

was subsequently soaked into tap water for 5-10 min and lastly into distilled water for 10 min. Tissue was then put into hematoxylin dye solution for 3 sec so that the dye reached nucleus. This prepare was subsequently soaked in tap water before soaking in distilled water, each for 5 min. Re-dying was proceeded by soaking the prepare into eosin dye for 2 min to color its cytoplasm. Prepare was re-soaked in distilled water for 5 min. The next part was dehydration or water removal to ensure durability by soaking prepare 2-3 times sequentially into 70, 80, 90, 95%, absolute I alcohol solution, then into absolute II and III alcohol solution for 1 min each. The final part was clearing by soaking the prepare into xylol I and xylol II solution for 1 min each then to xylol III for 3 min. Prepare was subjected to mounting, by which it covered using polyvinyl lactophenol as adhesive.

Statistical analysis: The experimental data were analyzed using one-way analysis of variance (ANOVA) (SPSS 20.0 Statistical Software Program for Windows). Significant differences among experimental mean values were assessed using Duncan's Multiple Range Test (DMRT) ($p < 0.05$).

RESULTS

Feed intake and body weight: Feed intake and body weight data are presented in Table 2. During intervention period, feed intake of diabetic rat was significantly different in all experimental groups, DM group had the highest feed intake. During 4 weeks intervention, feed intake in H, C4 and AR group were not changed. During initial period of intervention, body weight of all treatment groups was not significantly different. At 1 week after STZ-NA induction, DM, C4 and AR group showed 3.66, 5.66 and 1.50% body weight decrease, respectively. In subsequent period after 4 weeks intervention, C4 group had 6.95% increasing body weight, which was lower than those of AR of 9.19%. DM group had gradual body weight decrease during intervention period of 4.60%, whereas H group had increasing body weight of 18.34%.

Blood glucose: Blood glucose level during 4 weeks intervention in all treatment groups are presented in Table 3. DM group had increasing blood glucose level of 183.80%, continuously increased to 188.38% at final observation period. Those of C4 group was also increased of 184.81% after STZ-NA induction but decreased by 18.91% after fed with rice for 4 weeks. Similar pattern was also found in AR group, with 196.76% increasing blood glucose after induction but decreased by 55.07% after 4 weeks. H group had stable blood glucose level during intervention with average of 77.74 mg dL⁻¹.

Table 2: Feed intake and body weight during 4 week interventions

Parameters	H	DM	C4	AR
Feed intake (g)				
Week 0	16.33±0.31 ^c	19.05±0.27 ^a	16.90±0.53 ^b	16.45±0.56 ^{bc}
Week 1	16.22±0.63 ^b	19.02±0.14 ^a	16.02±0.45 ^b	16.36±0.33 ^b
Week 2	16.98±0.26 ^c	18.88±0.26 ^a	17.74±0.29 ^b	17.59±0.23 ^b
Week 3	16.93±0.53 ^b	19.07±0.17 ^a	17.31±0.30 ^b	16.90±0.21 ^b
Week 4	17.58±0.38 ^b	18.83±0.41 ^a	16.75±0.99 ^c	17.08±0.66 ^{bc}
Body weight (g)				
Week 0	186.33±3.01 ^a	188.33±6.77 ^a	187.83±3.43 ^a	188.17±6.55 ^a
Week 1	188.50±3.78 ^a	184.67±4.63 ^{ab}	182.17±2.99 ^b	186.67±5.05 ^{ab}
Week 2	206.17±3.87 ^a	180.83±4.87 ^d	186.83±3.25 ^c	196.17±4.71 ^b
Week 3	213.00±4.34 ^a	178.67±4.88 ^d	192.00±4.05 ^c	200.17±4.21 ^b
Week 4	220.50±4.13 ^a	176.17±5.19 ^d	194.83±3.87 ^c	203.83±3.76 ^b

Different superscript on the same line show a significant difference ($p<0.05$). H: Healthy rats+AIN 93M, DM: Diabetes+AIN 93M, C4: Diabetes+rice, AR: Diabetes+analog rice

Table 3: Blood glucose level (%) during 4 week interventions

Observations	H	DM	C4	AR
Before STZ-NA	76.23±0.89 ^a	73.16±1.81 ^b	73.91±2.36 ^b	72.47±1.93 ^b
Week 0	77.03±1.32 ^c	207.63±4.04 ^b	210.50±5.41 ^{ab}	215.07±8.60 ^a
Week 1	77.61±1.04 ^d	208.46±3.96 ^a	190.33±3.91 ^b	182.95±5.49 ^a
Week 2	78.06±1.22 ^d	209.71±3.56 ^a	186.49±2.72 ^b	150.91±4.07 ^c
Week 3	78.38±1.06 ^d	210.24±3.55 ^a	177.51±3.72 ^b	117.40±2.85 ^c
Week 4	79.12±1.10 ^d	210.98±3.82 ^a	170.69±2.72 ^b	96.62±2.92 ^c

Different superscript on the same line show a significant difference ($p<0.05$). H: Healthy rats+AIN 93M, DM: Diabetes+AIN 93M, C4: Diabetes+rice, AR: Diabetes+analog rice

Table 4: SCFA concentration (mmol L⁻¹) after 4 weeks intervention

SCFA concentration	H	DM	C4	AR
Acetate	28.59±7.58 ^a	25.56±3.33 ^a	33.54±9.78 ^a	34.27±4.21 ^a
Propionate	11.83±2.70 ^{bc}	8.73±3.26 ^c	13.96±2.86 ^{ab}	16.00±0.99 ^a
Butyrate	3.72±0.34 ^a	2.16±0.52 ^b	3.27±0.68 ^a	3.69±0.36 ^a
Total SCFA	44.14±9.58 ^{ab}	36.45±3.56 ^b	50.76±12.36 ^a	53.96±4.76 ^a

Different superscript on the same line show a significant difference ($p<0.05$). H: Healthy rats+AIN 93M, DM: Diabetes+AIN 93M, C4: Diabetes+rice, AR: Diabetes+analog rice

Table 5: Langerhans islets number after 4 weeks intervention

Langerhans islets diameter	H	DM	C4	AR
Small	2.80±1.79 ^{ab}	1.20±0.45 ^a	3.40±1.14 ^b	3.60±2.07 ^b
Medium	3.00±2.00 ^a	1.20±1.30 ^a	1.00±1.22 ^a	1.60±0.89 ^a
Large	4.00±3.16 ^{ab}	1.40±1.34 ^a	2.40±1.52 ^{ab}	5.40±2.30 ^b
Total	9.80±5.81 ^b	3.80±2.77 ^a	6.80±1.78 ^{ab}	10.60±3.58 ^b

Different superscript on the same line show a significant difference ($p<0.05$). H: Healthy rats+AIN 93M, DM: Diabetes+AIN 93M, C4: Diabetes+rice, AR: Diabetes+analog rice

Short Chain Fatty Acids (SCFA): The effect of diet treatment on the secum SCFA concentration are presented in Table 4. Cecum levels of SCFA acetate, propionate and butyrate varied significantly between the groups. AR group had the highest acetate, propionate, butyrate and total SCFA, followed by C4, H and DM group.

Langerhans islets cells: The Langerhans islets number after 4 weeks intervention are presented in Table 5. Hematoxylin-Eosin (HE) staining was conducted to qualitatively investigate general morphology of pancreas tissue after intervention. Among observation parameters on

pancreas prepare was the number of Langerhans islets, particularly average number of small, medium and large islets diameter, with approximate cell number criteria of 1-25, 26-50 and above 50 per islets, respectively³¹.

The highest Langerhans islets number after 4 weeks intervention was found in AR group (10.60±3.58), which also slightly higher than those of H group, though the latter had better small, medium and large Langerhans islets distribution. DM group had the lowest Langerhans islets number, significantly different ($p<0.05$) compare to other groups.

DISCUSSION

Experiment rats were diabetically induced using combination of streptozotocin (STZ), a diabetogenic agent with toxicity against pancreas β cells and nicotinamide (NA), a vitamin B₃ (niacin) derivate with antioxidant activity, to moderate toxic effect of STZ³². NA was reported by several studies for its ability to reduce small Langerhans islets damage by inhibition of DNA methylation³³. Induction using combination of STZ-NA caused attenuation of body antioxidant, significantly increases fat peroxide, hydroperoxide and carbonyl protein level in plasma, pancreas tissue and kidney³³. Sunarti³⁴ also noted that cellular oxidation-reduction imbalance coupled with increasing rate of free radical generation lead to the release of pro-inflammation cytokines. Interleukin (IL-6) is one of the cytokines with important role in DM pathogenesis released by fibroblast cells, endothelial cells, monocytes and adipose cells, induce insulin resistance in various tissue, such as liver and adipose.

STZ reduces glucose oxidation, insulin synthesis and secretion of β -cells as well as disrupt glucose transport and glucokinase activity. STZ-NA injection to induce diabetes on rats led to increase feed intake and reduction body weight of DM groups. The first symptom was caused pain suffered by rats decreased their appetite, which led to body weight reduction. After intervention, DM group had the highest feed intake compare to other groups. In diabetic rats (DM), high level of blood glucose cannot be utilized as energy source due to glucose uptake failure into muscle. This condition triggers glucose stored state, led to cell starvation or polyphagia³⁵. Impaired glucose metabolism-hunger of diabetic rats resulted in high feed intake of DM group.

On the other hand, since body failed to utilize glucose, energy sufficiency was supplied from break down of protein and fat in adipose tissue, hence body weight decrease. Thus, in DM group, despite higher feed intake, body weight was reduced. H group had normal carbohydrate metabolism and energy supply, thus prevented from polyphagia. During final period of intervention, feed intake of diabetic rats fed with rice and analog rice decreased. This was an indication of metabolism improvement due to feed which prevent polyphagia.

After 4 weeks intervention, body weight of rice-fed and analog rice-fed diabetic groups increased, similar to healthy rats, while those of standard feed group continuously decreased by 4.60%. One of the interesting findings of this study was that DM group had a significantly lower body weight gain throughout the experimental period. This indicated carbohydrate metabolism improvement on diabetic

group with rice and analog rice diet. The later had better improvement closer to healthy rats. The improvement was also marked by blood glucose reduction. These results showed that high RS and dietary fiber intake has a positive impact on diabetes.

Blood glucose decrease after 4 weeks obtained by C4 and AR group were 18.91 and 55.07%, respectively, while H group had stable blood glucose level during intervention at average of 77.74 mg dL⁻¹. It was indicated that analog rice diet had the highest hypoglycemic property, followed by rice consumption. It was possibly caused by resistant starch and dietary fiber as noted by previous research that analog rice made from mocaf, arrowroot and kidney beans contains high fiber and resistant starch²³. They cannot be digested in small intestine, fermented in colon, then generate Short Chain Fatty Acid (SCFA) which predicted as the mechanism that improve insulin sensitivity, led to increasing glucose uptake.

In colon, RS fermentation by microflora generates Short Chain Fatty Acid (SCFA) of acetate, propionate and butyrate. In this study, total SCFA concentration of AR group (53.960 mmol L⁻¹) and C4 group (50.762 mmol L⁻¹) was significantly higher than those of DM group (36.452 mmol L⁻¹) and even H group (44.142 mmol L⁻¹). SCFA reduce blood glucose through several mechanisms. Canfora *et al.*³⁶ and Gao *et al.*³⁷ explained that acetate and propionate, two main SCFA products of RS fermentation, were able to increase buffering capacity which induce reduction of muscle's fatty acid, hence reduce muscle's lipid storage capacity and improve insulin sensitivity. Moreover, Luo *et al.*³⁸ also mentioned that high level of free fatty acid inhibit glucose utilization in muscle tissue, exacerbate insulin resistance. Other research explained that increasing rate of SCFA concentration in human body reduces free fatty acid, thus improve insulin sensitivity^{39,40} and subsequently improves glucose response.

In medium condition of diabetes, the number of β cells is drastically reduced so that they cannot be found in chronic level. However, α -cell can still presence in peripheral part of Langerhans islets. Chronic hyperglycemia results in glucotoxicity against β -cells which lead to its dysfunction and reducing mass, finally reduces insulin secretion⁴¹. Nugent *et al.*⁴² reported that the last stage of type 2 diabetes is marked by reduction of β -cells mass, intra-islet amyloid (IIA) deposition and fat deposition into Langerhans islets.

Pancreas tissue belongs to AR group had higher Langerhans islets number among all groups which indicated inhibition of β cells damage in treatment groups with highest resistant starch diet. Keenan *et al.*^{43,44} reported that high resistant starch consumption might promote glucagon

like-peptide-1 (GLP-1) gene expression in intestine and blood of diabetic rats. Propionate as one of SCFA fermentation product from resistant starch is also able to increase GLP-1 expression⁴⁵. GLP-1 plays the role as incretin hormone which inhibit glucagon secretion, mediates glucose-dependent insulin secretion through receptor in β -cells as well as promotes β -cell mass improvement through proliferation and apoptosis inhibition¹⁰. This report signifies that high resistant starch consumption might improve or inhibit β -cells damage in diabetes.

CONCLUSION

Consumption of analog rice (AR) made from mocaf, arrowroot and kidney beans showed hypoglycemic effect with blood glucose level decrease of 55.07%. Total SCFA concentration of groups fed with analog rice (AR) and rice (C4) was 53.96 and 50.76 mmol L⁻¹, respectively. Analog rice (AR) group also had higher Langerhans islets of 10.60 than rice fed (C4) group of 6.80. The results indicated that RS and dietary fiber was responsible to glucose reduction effect by analog ricediet through SCFA as RS fermentation product in colon.

SIGNIFICANCE STATEMENT

This study discovered that consumption of high resistant starch and dietary fiber analog rice made from mocaf, arrowroot and kidney beans decreased blood glucose level, which became a promising nutritional therapy for diabetes mellitus patients. The result of this study was expected to help researchers to uncover the critical areas of food applications using analog rice. Thus, new informations on analog rice contribution in nutritional therapy can be obtained and used in practical way.

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