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Effect of Soybean-based Food Supplement on Insulin and Glucose Levels in Type 2 Diabetes Mellitus Patients

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ABSTRACT

Administration of soy may stimulate increase levels of glucagon-like peptide 1 (GLP-1). Giving soy supplements to people with diabetes can reduce blood glucose levels significantly. However, no studies have shown this reduction effect of glucose level back to normal levels. This study aimed to determine the blood insulin and glucose levels after supplementation of soybean-based food supplements in patients with type 2 diabetes mellitus (T2DM) and the sequencee of DNA of GLP-1 gene. This experimental study was a randomized, treatment controlled; open clinical trial study conducted by comparing the group treated with soybean supplement products containing 18g protein and the group of placebo. Seventy-six, subjects with T2DM were recruited from Muhammadiyah Gamping Hospital and PERSADIA Gymnastics, Yogyakarta, based on the inclusion and exclusion criteria. All subyects were grouped randomly into the supplement group and placebo group. Blood sampling was drawn at 8h fasting, 2h after administration of supplement and 2h after administration of 75g glucose. The determination of blood insulin and glucose level and DNA sequencing were performed. Blood insulin level 2h after supplement administration (mean±SEM) increased 5.3±0.8μIU/mL (n=37) while placebo decreased 0.9±0.4μIU/mL (n=39) which was significantly different (p<0.05). In both groups blood glucose levels incresed as much as 130.0±11.5mg/dl (n=37) and 146.7±8.2mg/dL (n=39) (p>0.05), for the supplement treated and placebo groups, respectively. DNA sequencing shows a nucleotide variation of GLP-1 (37 amino acid) in Javanese T2DM. It may be concluded that administration of soybean-based supplements containing 18g protein increased blood insulin levels and decreased blood glucose levels.

Keywords: soybean supplements, insulin, blood glucose, type 2 diabetes mellitus, GLP-1 gene

INTRODUCTION

The International Diabetes Federation (2015) estimates that people with diabetes mellitus (DM) in the world reached 415 million in 2015 and will increase to 642 million by 2040. Ten countries with the largest population of DM sufferers in the world are India, China, America, Indonesia, Japan, Pakistan, Russia, Brazil, Italy and Bangladesh (Khardori, 2016). DM is a risk factor for cardiovascular disease. It may be controlled by treatment with anti-diabetic drugs and dietary restrictions (Balitbangkes, 2013). DM management is conducted by the efforts of non-pharmacologic and pharmacologic treatment administration. Non-pharmacological interventions involve applying healthy lifestyles and pharmacological interventions by administering drugs orally and/or injections (PERKENI, 2015). Encouragement of herbal medicine (*jamu*) research based on health services promoted by Indonesian government

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since 2010 has increased the use of herbal medicine for patient services by the medical profession. This approach requires support of scientific studies on efficacy and safety the herbal medicines (Delima *et al.,* 2012; Herman *et al.,* 2013).

Two hours after administration of soy supplements to people with T2DM showed postprandial glucose levels were significantly lower compared to controls (p<0.05) (Chang *et al.,* 2008; Kwak *et al.,* 2010). Soy milk can reduce blood glucose levels in people with diabetes mellitus (Cahyono, 2011). Giving soy milk for 14 days decreases fasting blood glucose levels in prediabetes women (Sinaga & Wirawanni, 2013). Soybean supplements can reduce blood glucose levels in diabetic mice and increase white blood cells compared to the normal saline control group (Sada *et al.,* 2013). Chang *et al.* (2013) reviewed hundreds of papers that discussed the structure, activity, and mechanisms of the actions of various plant chemical compounds for the treatment of type 2 diabetes mellitus (T2DM). Among plants that have the effect of improving the condition of hyperglycemia in the models and in patients those with T2DM are soybean plants. Giving soy flour fortified bread did not have a significant effect on the metabolic profile including fasting glucose levels, cholesterol, triglycerides, LDL cholesterol, and HDL cholesterol compared to the usual diet in diabetic patients (Moghaddam *et al.,* 2014). Administration of 280mL soy milk per day for 14 days can reduce blood sugar levels of people with type 2 diabetes mellitus compared to the placebo control group (Baequny *et al.,* 2015). The habit of consuming phytoestrogens, especially in the form of whole food sources such as soybeans and flaxseed, can be considered as a healthy diet for prevention and management of type 2 DM (Talaei, 2015). Isoflavone intake is associated with a lower risk of T2DM in US people who usually consume small to moderate amounts of soy foods (Ding *et al.,* 2016). Consumption of soy and cow's milk induces a decrease in similar glycemic responses (Sun *et al.,* 2016). Supplementation of soy protein more than 6 months significantly reduces Fasting Plasma Glucose, LDL-Cholesterol in T2DM and metabolic syndrome (Zhang *et al.,* 2016). High intake of soybeans and isoflavones can reduce the risk of lower type 2 diabetes in Vietnamese adults (Nguyen *et al.,* 2017). Administration of 15g of soy protein with 66mg of isoflavones improved significant glycemic control compared to 15g of soy protein without isoflavones (Sathyapalan *et al.,* 2017). The combination of soybean and ginger rhizome extract reduced blood glucose levels better than single soybean seed extract in diabetic mice (Purnomo, 2018). Soy products may be associated with a lower risk of T2DM but requiring further studies that focuses on the dose-response and mechanism of action (Li *et al.,* 2018).

Treatment of soybeans containing 50g of protein increased GLP-1 levels by approximately 6pg/mL at 30min and remained at almost the same level for up to 2-3h after administration with a significant difference compared to glucose in overweight (Bowen *et al.,* 2006). The administration of macronutrients containing soybeans increase levels of glucagon-like peptide 1 (GLP-1) (Douglas *et al.,* 2015). (GLP-1) is released by intestinal L-cells after stimulating nutrients and regulating glucose levels by stimulating insulin secretion (Janus *et al*., 2019). Genistein stimulate secretion of insulin through cAMP-dependent protein kinase pathway (Liu *et al.,* 2006). Amino acids play an important role in stimulating GLP-1 (Mansour *et al.,* 2013). Bioactive of soy hydrolyzates stimulate an increase in GLP-1 (Tulk *et al.,* 2014). Genistein activates AMPK so that it can reduce insulin resistance (Chang *et al.,* 2013). AMPK alters the transcription and secretion of GLP-1 in intestinal secretin tumor cell line (STC-1) culture (Jiang *et al.,* 2016). Soy milk increases incretin levels (GLP-1 and GIP) which have an effect on reducing blood glucose levels (Sun *et al.,* 2016).

Although the effect of giving soybeans in reducing blood glucose levels in people with T2DM has been demonstrated an adequate clinical evidence is needed for its therapeutic use in medicinal treatments. Therefore, the study was conducted to determine how much insulin and blood glucose levels is altered in T2DM by administration of soy-based supplements.

MATERIALS AND METHODS

This experimental study was performed by randomized, controlled treatment using an open clinical trial design comparing treatment groups administered soybean supplement products containing 18g of protein to control groups given placebo (water) in patients with T2DM. The subjects of the study were 76 people with T2DM recruited from The Hospital of PKU Muhammadiyah Gamping, Yogyakarta and the PERSADIA gymnastics group who fulfilled the inclusion and exclusion criteria. All subjects were willing to take part in the study by completing informed consent forms. The participants were

grouped randomly into 2 groups of supplement treatment and placebo control. Blood samples were drawn after 8h fasting and at 2h after administration of supplement product and or placebo. The third blood sampling were taken 2h after 75g glucose loading for measuring insulin and blood glucose. Levels and DNA sequencing of the the gene of GLP-1 (37 amino acids).

The subjects were selected based on the inclussion and the exclussion criteria.

Confounding variables include age, body mass index, lipid profile, and drug use were recorded. Age >45 years is a risk factor in the development of diabetes mellitus, obesity especially central obesity is closely related to the development of type 2 DM can be measured by body mass index. Obesity is also closely related to a person's lipid profile so it needs attention for not to interfering to the variables measured. The use of insulin or oral hypoglycemic agents that may interfere glucose level should be controlled. Therefore, the blood sampling and the administration of supplement were conducted at the time after the patients were fasting for at least 10 hours or after the last dose of anti-hypoglycemic agent and or insulin. Efforts to minimize the influence of confounding factors were randomly allocated subjects into the study group or where possible avoiding these factors in the study.

Instruments

The equipment used in the study were Venopuncture and injection syringes used for taking blood sampling, EDTA tubes used for blood collection, cooler boxes, Elisa readers for insulin testing, and GENIUS clinical laboratory devices for blood glucose levels.

Material

A factory-made soy supplement product is obtained from market. Thirty five (35) g of powder per serving mixed with 250mL of water with a shaker to obtain a protein content of 18g. To measure insulin levels an ELISA kit was used from CALBIOTECH® while the blood glucose level examinations used Glu Reagent Kit from Shenzhen Genius Electronic Co. Ltd. DNA template preparation of GLP-1 gene firstly was conducted by DNA isolation used Blood Genomic DNA Extraction Mini Kit With Proteinase from Favorgen Biotech Corp. Polymerase Chain Reaction (PCR) was performed used upstream oligonucleotide primer (forward) 5'CTCGCCTTCCTCGGCC3' and downstream oligonucleotide primer (reverse) 3'GAATAACATTGCCAAACGTCACG5' refers to coding region accession no NC_000002.12 according to exon 4 glucagon preproprotein DNA, go taq® green master mix 2x1.25mL and nuclease free water.

Treatment of the subjects:

At the time of the study, subjects were available to the Clinical Laboratory of The Hospital of PKU Muhammadiyah, Gamping, Yogyakarta, for fasting blood collection at 06.00-07.00AM. Subsequently, all subjects were given either a glass of soybean supplement or a glass of water to the treatment group or to the placebo group, respectively. The suplement were given at 10h after the last dosing of antidabetic agent. Two hours after taking supplement or placebo, the second blood sampling was taken. The first and the second blood samples were analyzed for insulin and glucose level. After the second blood sampling, all the subjects took 2 pieces of white bread and drank a glass of sweet milk equivalent to 75g of glucose. Two hours after eating white bread and drinking sweet milk blood samples were drawn again for the third time to measure the 2h postprandial blood glucose as glucose tolerance tests. Determination of insulin was performed by ELISA in the Biomedical Laboratory of FM-PHN UGM. The GLP-1 (37 amino acid) gene sequencing was performed by 1st BASE DNA Sequencing Division of First Base Laboratories Sdn Bhd Malaysia.

Polymerase Chain Reaction (PCR) for c-DNA of GLP1

The PCR protocol was put in 30µL go taq® master mix, 2µL primer forward, 2µL primer reverse, 22µL nuclease-free water (NFW) and 4µL DNA template into 2m PCR tubes and then place the tubes in heat block of thermo cycler and proceed with the thermal cycler setting: 95°C 5min for denaturation, 40 cycles (95°C 1min, 57°C 1min annealing temperature, 72°C 1min), 70°C 5min for extension and 4°C indefinitely, finally the PCR result was observed by gel electrophoresis.

PCR was conducted in biomedical laboratory of Faculty of Medicine - Public Health and Nursing (FM-PHN) UGM. Subsequently, The PCR results were sent to First Base Laboratories in Malaysia. Cycle sequencing, purification after cycle sequencing and capillary electrophoresis were conducted at the 1st BASE DNA Sequencing Division of First Base Laboratories Sdn Bhd Malaysia.

Table I. Characteristic of the subjects

Variable		Suplement Group	Placebo Group	Statistic significans
	Sex ♂/♀	18/19	18/21	P>0.05
	Age (years)	56.6±1.3	57.6±1.4	P>0.05
	Length of diagnose of DM (years)	7.8±0.0	7.9±1.1	P>0.05
	Body height (cm)	159±0.01	158±0.01	P>0.05
	Body weight (kg)	64.6±2.3	64.56±1.8	P>0.05
	BMI	25.0±0.6	25.1±0.6	P>0.05
Blood presure	Sistolik	140.3±2.	144.64±2.7	P>0.05
	Diastolik	77.2±1.5	80.4±2.2	P>0.05
	Cholesterol level	219.8±11.8	19.24±10.1	P>0.05
	HDL	46.5±1.7	47.7±1.8	P>0.05
	LDL	126.1±7.1	113.4±7.5	P>0.05
	Triglycerides	197.6±20.5	218.9±30.0	P>0.05
Drugs used	OHA	26	24	P>0.05
	OHA + insulin	11	15	P>0.05

*Numerik data were tested by t-test Man Withney; *Categorical data were tested by Chi-square; OHA = oral hypoglycemic agent

Table II. Blood Insulin Levels glucose

Group	Mean±SEM of Blood Insulin Level (µIU/mL)			Mean±SEM of Blood Glucose Level (mg/dL)		
	Supplement n=37	Placebo-H2O n=39	p-value	Supplement n=37	Placebo-H_2O n=39	p-value
Fasting	11.8±2.5	14.9±2.3	>0.05	192.1±13.7	189.4±11.2	>0.05
Post supplementation	17.0±2.8	14.0±2.3	>0.05	221.0±14.9	181.1±11.6	<0.05
Increase	5.3±0.8	(0.9±0.4)	<0.05	351.0±19.8	327.5±14.3	>0.05

SEM: Standard Error Mean

Capillary electrophoresis results in the form of AB1 files analyzed by BioEdit Sequence Alignment Editor for Windows. Sequencing of GLP-1 (37 amino acid) gene used Applied Bio-systems automated DNA sequencing which consists of 5 step includes DNA template preparation, cycle sequencing, purification after cycle sequencing, capillary electrophoresis.

RESULTS AND DISCUSSION

There are no significant differences were found between the placebo and the supplement groups in the term of variables which may affect blood glucose level and or insulin level such as age, sex, body weight and BMI, profile lipids, the length of diagnose of DM. Not any subjects involved was categorized as obesity. Some of the subjects are used to administer oral hypoglycemic agent or insulin or combination of the two, but at the time of having supplementation was assumed that the effect of all types of anti-diabetic agent were neglected (Table I).

Fasting insulin levels in the supplement group were 11.8±2.5µIU/mL while the placebo group was 14.9±2.3µIU/mL, and the difference between them was statistically insignificant (Table II) Insulin levels in the treatment group after supplementation with soybean-based supplements increased to 17.0±2.8µIU/mL while the placebo group levels was still to be 14.0±2.3µIU/mL. The increase in insulin levels in the supplement group was 5.3±0.8µIU/mL and the decrease in the placebo group insulin level was 0.9±0.4µIU/mL. The changes of insulin level between the two groups was significant different (p<0.05).

The average fasting blood glucose levels in both the supplementary and placebo treatment groups were above the normal value of fasting glucose levels according to PERKENI, 2015 (>126mg/dL) (Table II).

Table III. Increase in blood glucose levels after supplementation and 2h postprandial.

Group	Mean±SEM of Increase Blood Glucose Level (mg/dL)		
	Supplement n=37	Placebo-H_2O n=39	p-value
Post Treatment (supplementation)	28.9±6.6	(7.8±5.5)	<0.05
2h postprandial	130.0±11.5	146.7±8.2	>0.05

SEM: Standard Error Mean

Tabel IV. Blood Insulin Level (µIU/mL) and Blood Glucose Level (mg/dL). Subject treated with insulin excluded

Group	Blood Insulin Level (µU/mL			Blood Glucose Level (mg/dL)		
	Suplement group N=26	Placebo N=24	p-value	Suplement group N=26	Placebo N=24	p-value
Fasting	9.5±2.9	13.5±3.0	>0.05	212.3±15.8	182.3±13.8	>0.05
Post Suplement	15.2±3.1	12.9±2.9	>0.05	237.3±18.8	170.8±11.0	<0.05
2h postprandial				355.3±25.5	304.5±15.0	>0.05
Increase	5.7±1.0	(0.6±0.5)	<0.05			

Tabel V. Increased of Blood Glucose Level (mg/dL) after supplementation and 2h post prandial. Subject treated with insulin excluded

Group	Suplement group N=26	Placebo N=24	p-value
Post Suplement	25.0±7.7	(10.8±7.5)	<0.05
2h post prandial	118.0±10.7	146.7±8.2.0	>0.05

However, there is no significant differences of the blood glucose level between the two groups (p>0.05). After supplementation of soybean supplement, blood glucose levels increased to 221.0±14.9mg/dL (mean±SEM) while in the placebo group there was a decrease to 181.1±11.6mg/dL (mean±SEM). The mean of the two groups were significantly difference (p<0.05). The blood glucose levels after treatment of the supplement increased by 28.9±6.6mg/dL compared to the placebo group in which the blood glucose level decreased by 7.8±5.5mg/dL (p<0.05) (Table III). Blood glucose level in supplement group increased may be caused by serving of supplement of soybean-based contain approximately 11g carbohydrate which could be metabolized into glucose.

Soybean ingredient contain protein and isoflavon of genistein. Protein in gastrointestinal tract were brook down into amino acid. Amino acid, glucose and genistein have an effect to stimulate secretion of GLP-1 that stimulate beta cell pancreas to release insulin. Therefore, after given soybean supplement in the supplement group insulin levels should increase and accordingly glucose levels decrease. However in this study the soybean suplement resulted in the increase of glucose levels (Tabel II). It could not be explained the logic of this phenomena. Administration of one serving of supplements made from soybeans containing 18 grams of protein can be given as a substitute for one meal without concurrent anti-diabetic drugs if the basal glucose level is normal because the increase in blood glucose levels after administration is quite low with an average increase of 28.9±6.6mg/dL.

The average postprandial glucose level of the supplement group was 351.0±19.8mg/dL compared with the placebo group of 327.5±14.3mg/dL and this difference was not significant (p>0.05). Blood glucose levels after 2h postprandial in the supplement group appeared to be higher than the placebo group because at the beginning in the supplement group showed higher glucose level than in the placebo group (Table III). After 2h postprandial with 75g glucose loading and after soybean supplement treatment showed that in the supplement group there was an increase in blood glucose levels by 130.0±11.5 mg/dL, this was comparable to the increase the glucose level in the placebo group, which the increase was 146.7±8.2 mg/dL (p>0.05).

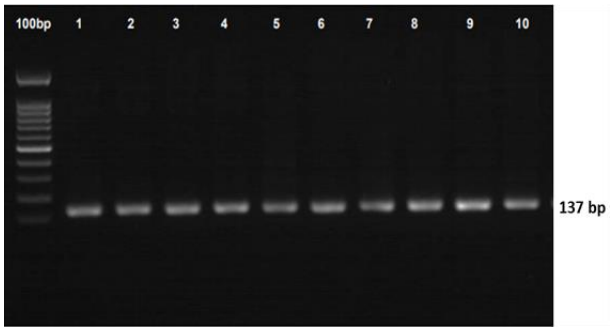

Figure 1. Gel electrophoresis PCR of DNA of GLP-1 gen

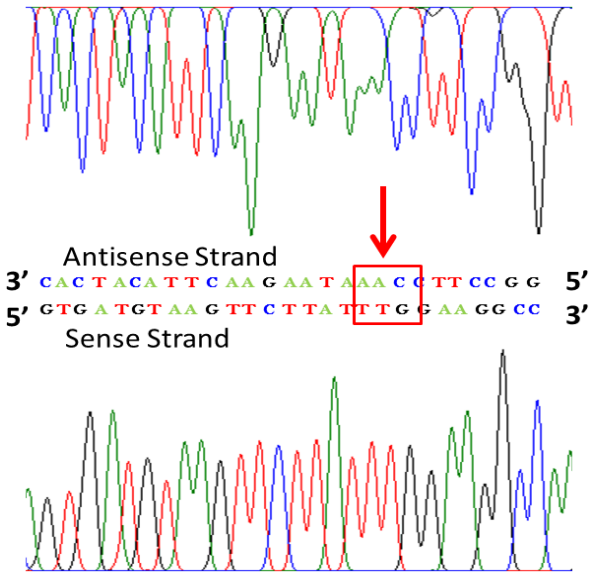

Figure 2. the electropherogram of the result of capillary electrophoresis shows the normal sequence (wild type) of the gene of GLP-1 refers to coding region accession no NC_000002.12, the DNA sequence of antisense strand around Leu111 is 5' GGCCTTC**CAA**ATAAGAACTTACATCAC 3' while the sense strand is 5' GTGATGTAAGTTCTTAT**TTG**GAAGGCC 3'.

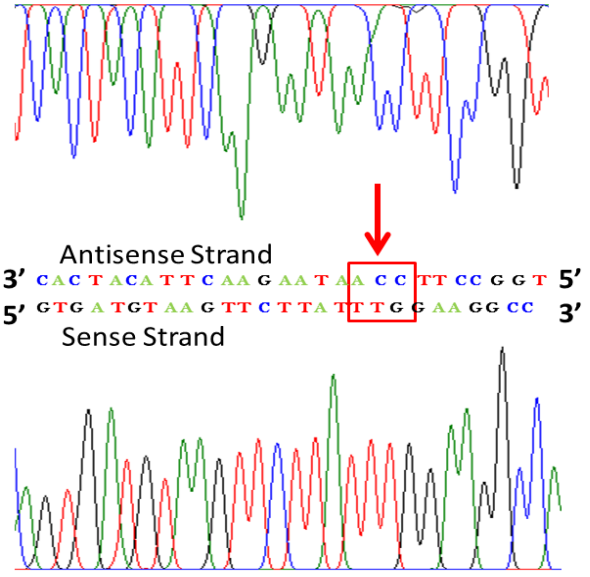

Figure 3. the electropherogram of the result of capillary electrophoresis shows the abnormal sequence of the gene of GLP-1, in the red box it visible to be lost one nucleotide (Adenine) compare to figure 1. In this electropherogram sequence of the antisense strand change to 5' GGCCTTC**C-A**ATAAGAACTTACATCAC 3' while the sense strand still unchanged it same with the reference 5' GTGATGTAAGTTCTTAT**TTG**GAAGGCC 3'.

This results indicate that soybean supplementation have no effect in increasing blood glucose level for diabetic subjects. Most of blood glucose levels in the two groups were more than 200mg/dL, while some were below 200mg/dL. But the NNT (Number Needed to Treat) value between the supplement versus the placebo group was statistically not significant. Therefore, the use of soy-based supplements containing 18g of protein cannot be recommended as a specific therapy to control blood glucose levels in patients with T2DM. However, its use as a substitute for breakfast or dinner can still be suggested based on the average increase in glucose after the treatment of soy-based supplements, which was only 28.9±6.6mg/dL.

Table VI. Difference in blood insulin levels and blood glucose levels in soy supplementation based on GPL-1 genetic variations of antisense strand NC_000002.12: g.162145600delA.

Group	Average ±SEM difference blood insulin level (µU/mL)			Average ±SEM difference blood Glucose Level (mg/dL)		
	Suplement	**Placebo**	**p-value**	**Suplement**	**Placebo**	**p-value**
AA (n)	7.2±2.9 (6)	1.1±0.9 (10)	<0.05	133.6±36.5 (6)	153.4±18.7	>0.05
DelA (n)	4.9±0.8 (31)	(-0.9±0.5) (27)	<0.05	129.4±12.2 (31)	143.5±9.7 (27)	>0.05

SEM: Standard Error Mean blood insulin level, η^2=0.41 p<0.05, Mean blood glucose level, η^2=0.02 p>0.05,

The changes of blood insulin and glucose level in the subject who do not use insulin during routine therapy (Table IV and V). It can be seen that the changes of the both parameter after supplementation and or after 2h-meal (2pp) exhibited similarity with the changes of those in all subjects involved, both subject with and without insulin during DM therapy. The result of DNA isolation of GLP-1 gene which amplified by PCR shows an image of amplification of DNA of GLP-1 (37 amino acid) gene as band of 137 bp length of PCR product on gel electrophoresis (Figure 1).

The sequencing results of the gene of GLP-1 (37 amino acid) showed a difference of nucleotide sequences in the antisense strand than a normal reference which is CAA>C-A found in a most subject which coincided with the 111th amino acid of glucagon preproprotein, leucine (Leu111). The Leu111 mRNA coding which is the sense strand is TTG. The normal double-stranded DNA according to accession number NC_000002.12 precisely around the Leu111 of glucagon preproprotein (Figure 2).

In this study, it was found that GLP-1 (37 amino acid) sequencing examination showed abnormalities, antisense strand DNA sequences that coincided with the 111th amino acid of glucagon preproprotein precisely at the chromosome position NC_000002.12:g.162145600delA. Normal reference sequence of antisense strands nucleotide number 162145599-162145601 is 5'CAA3' but the results of the examination found the subjects shows 5'C-A3' sequences (Figure 3).

There has never been reported a single nucleotide variation (SNV) of deletion (CAA>C-A) at the position of the amino acid Leu111 from the glucagon preproprotein gene. The NCBI Reference shows that the nucleotide variation associated with the Leu111 amino acid that exists is substitution on antisense strand CAA>CAG which it sense strand TTG>CTG, it SNV is synonymous encoding leucine amino acids and its occurrence is very rare with minor allele frequency (MAF) less than 0.0001. Deletion at the position NC_000002.12:g.162145600delA in diabetics patients of the Javanese may relate to the changes of insulin levels need require more extensive and more rigorous study, so far, these single nucleotide variations have not been reported at all. However, if indeed the deletion exists even though it has not been disturbed clinically so the absence of nucleotides on one side of the DNA strand will make an unstable DNA. This condition allows other nucleotides to be occupied or a pair of nucleotides will disappear and affect the protein product when DNA transcribed and then translated in the process of protein synthesis. Finally, the amino acid composition of protein products may change and may affect its function clinical manifest as a disorder or disease. Theoretically adenine deletion on antisense strand position NC_000002.12:g.162145600delA may change amino acid sequence (DVSSYlegqaakefiawlvk...(+55 amino acids)...*->DVSSYwkaklprnsllgw*) so it will influent the GLP-1 function. In this study, the administration of supplement change insulin level (η^2=0.40 p<0.05), the changes may/may not relate to genetic variation. The increase in blood insulin levels after the soybean supplement treatment appeared to be higher in the normal genotype group of 7.1±2.9µIU/mL (n=6) compared to the abnormal (mutant) genotype group of 4.9±0.7µIU/mL (n=29) the correlation η^2=0.41 statistically significance (p<0.05). More clearly with respect to subjects with normal and abnormal DNA sequences to insulin levels and blood glucose levels both in the treatment group of soybean supplements and the control group can be observed (tables VI).

CONCLUSION

From the study, it can be concluded that there is a relationship between the administration of soybean supplements and the changes of blood glucose and insulin levels. Genetic variation in normal genotypes of GLP-1 at the position of the antisense strand NC_000002.12: g.162145600delA may relate to the cganges blood insulin levels. The administration of soy-based supplements containing 18g of protein increased blood insulin levels and suppressed blood glucose levels but cannot reversed back to normal levels. Although it cannot be used as specific therapy in Type 2 diabetes mellitus, one serving supplement as a substitute for a meal can be recommended without anti-diabetic medications if the basal glucose level is normal.

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