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The effect of fermented tempeh aerobic anaerobic (FETAA) on *pancreatic duodenal homeobox 1* (Pdx1) gene expression and HOMA-beta index in diabetic mice

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ABSTRACT

Background: Diabetes is a result of oxidative stress which causes the impaired function of pancreatic beta-cells. Fermented tempeh aerobic anaerobic (FETAA) containing gamma-aminobutyric acid and isoflavones can reduce oxidative stress in diabetes.

Objective: The aim of this study is to evaluate FETAA in improving pancreatic β-cell function in diabetic mice.

Methods: Twenty streptozotocin-induced diabetic mice, divided into four groups (n = 5 each group): DM, DM + FETAA 10 mg/100 g BW, DM + FETAA 20 mg/100 g BW, DM + FETAA 40 mg/100 g BW as well as normal group (n = 5). DM mice were treated with FETAA for 21 days. Fasting glucose was determined using the GOD-PAP method, while insulin level was determined by ELISA. The homeostasis model assessment of β-cell function (HOMA-β) was calculated using the HOMA2 calculator, and the Pdx1 mRNA level was determined by Real Time-PCR.

Results: The DM mice group treated with FETAA had lower glucose levels than the DM mice group. FETAA dosage of 40 mg/100 g BW was able to reduce the highest blood glucose levels ($p<0.05$). DM mice group treated with FETAA had higher levels of insulin and HOMA-β than the DM mice group ($p <0.05$). Treatment of FETAA 10 mg/100 g BW produced the highest insulin content of 57.44 ± 8.132 pmol/L, while treatment of FETAA 40 mg/100 g BW had a HOMA-β value of 72.86 ± 21.85%. Pdx1 mRNA expression in group FETAA-treated DM mice was higher than the DM mice group, although it was not statistically significant ($p> 0.05$).

Conclusion: FETAA could improve HOMA-β, blood glucose levels, but did not affect Pdx1 mRNA expression.

Keywords: fermented tempeh aerobic anaerobic, gamma aminobutyric acid, HOMA-beta, pancreatic duodenal homeobox 1, type 2 diabetes mellitus

Introduction

Diabetes mellitus (DM) has the third rank cause of death in Indonesia [1]. The number of DM patients has tended to increase in recent years. According to the International Diabetes Foundation (IDF), it is estimated that in 2035 the number of DM sufferers will be around 382 million worldwide [2]. More than 80% of DM patients are found in developing countries and 60% are found in Asia [3]. The majority of people with diabetes, among 90-95%, are patient of type 2 diabetes and the prevalence rate of patients tends to increase in developing countries such as India, Brazil, Pakistan and Indonesia [4]. Treatment of type 2 diabetes mellitus is long-term, and has various side effects, such as long-term use of insulin secretagogues can damage pancreatic β-cells due to overstimulation [5].

Regeneration approach therapy is required in diabetes mellitus therapy. The regeneration of pancreatic β cells can be achieved through two mechanisms, namely self-replication of pancreatic β

cells and conversion of α cells to β cells. Regeneration of pancreatic β cells in diabetes can occur through stimulation of several transcription factors, signal pathways, and mediators [6]. Gamma-aminobutyric acid (GABA) is known to be a substance that has the ability to repair and protect pancreatic β cells [7]. Fermented tempeh aerobic anaerobic (FETAA) is fermented soybean products that are made by using a combination of aerobic and anaerobic fermentation methods. An anaerobic fermentation could increase the GABA content compared to traditional tempeh [8]. The combination method could increase the isoflavone content compared to traditional tempeh [9].

Pancreatic duodenal homeobox 1 (Pdx1) is the main transcription factor that plays a role in the process of organogenesis and regeneration of the pancreas [10]. Homeostatic model assessment (HOMA) is a formula to assess the function of pancreatic β-cells by correlating blood glucose and insulin balance [11]. The aim of this study is to evaluate FETAA in the improvement type 2 diabetes mellitus mouse by evaluating expression of the Pdx1 and HOMA-β index.

Methods

Preparation of fermented tempeh aerobic anaerobic

Soybeans (*Glycine max* L.) Anjasmoro varieties originated from Indonesia were purchased. *Rhizopus* FNCC 6010 inoculum was obtained from the Food and Nutrition Culture Collection, IUC Food, and Nutrition Gadjah Mada University. About 1000 g of unhulled soybeans were soaked with tap water at room temperature for 24 hours. Then, soybeans were cooked with water that was used for soaking for 15 minutes in boiling water. The cooked soybeans were cooled down to 27°C and then subsequently dehulled the soybeans by mixing vigorously the suspension manual. The dehulled soybeans were soaked again in fresh tap water by following the first procedure.

Once cooled to room temperature, the cooked soybeans were fermented by inoculating a spore suspension (25 mL of spore suspension) into the cooked soybeans (1 kg). About 120 g of inoculated beans were packed into perforated polyethylene plastic bags and incubated aerobically for 20 h at 37 °C (traditional tempeh). Anaerobic incubation was then conducted by transferring all the packed soybeans into Oxoid AnaeroJar 2,5 L containing AnaeroGen 2,5 L (Thermo) and incubated for 23 h at 37 °C. Subsequently, the FETAA was then lyophilized using the BenchTop Pro with Omnitronics-Freeze dryer, USA, and then ground into a powder.

Ethical clearance

The animal experiments were approved by the Medical and Health Research Ethics Committee (MHREC) of Faculty of Medicine Gadjah Mada University (authorization number Ref. No: KE/FK/0335/EC/2019) under the recommendations for handling animals for research.

Animal model

A total of 25 male Balb/c mice (8–10 weeks old; 26–30 g) were housed in polycarbonate cages with an artificial 12-h light/dark cycle at a constant temperature (25 °C). The mice were fed an AIN-93 M diet (D10012M-Research Diet, USA). Mice were acclimatized for seven days; then mice were randomly divided into non-diabetic (n = 5) and diabetic (n = 20) groups. The diabetic mice model was induced by a single injection of streptozotocin (STZ) (50 mg/kg BW in 0,1 M citrate buffer, pH 4,2; Sigma, St. Louis, MO, USA) into the peritoneum on two consecutive days. Nicotinamide (NA, 120 mg/kg BW; Sigma, St. Louis, MO, USA) was dissolved in saline and injected intraperitoneally 15 min before the administration of STZ [12]. The non-diabetic mice were injected with citrate buffer or saline alone. After five days, only STZ/NA-treated mice that exhibited a fasting blood glucose level of ≥196 mg/dL were used in the study.

The diabetic mice were randomly subdivided into four groups each group contained five mice: the diabetic group (DM), the diabetic given FETAA

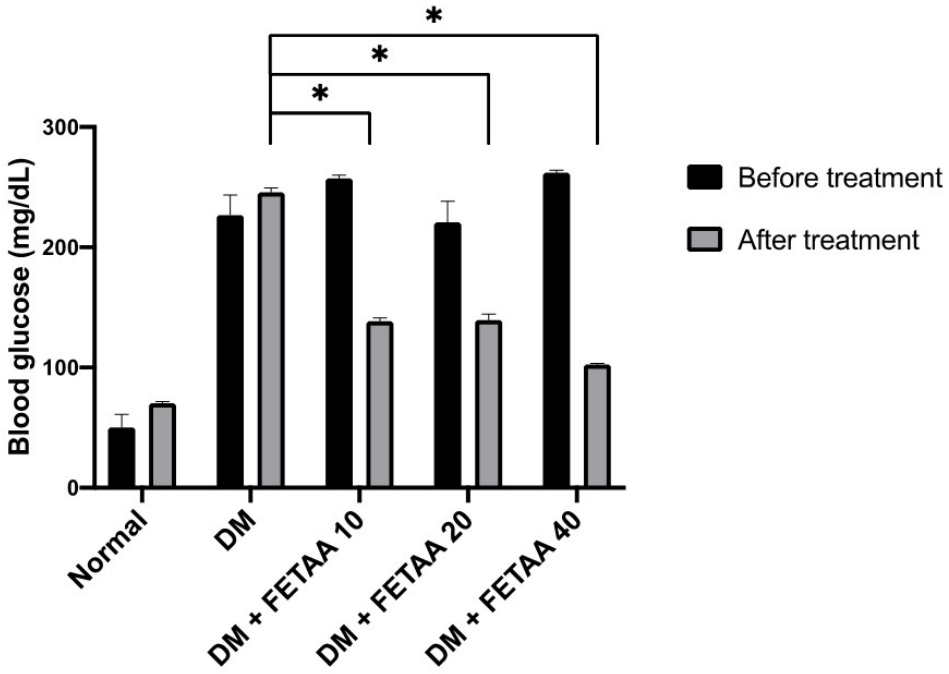

Figure 1. Total fasting blood glucose of treatment groups of mice. Statistical analysis used the One-way Anova test followed by the Howell Games post hoc test with * $p<0.05$ stated as significantly different

10 mg/100 g BW (DM + FETAA 10), the diabetic given FETAA 20 mg/100 g BW (DM+FETAA 20), the diabetic given FETAA 40 mg/100 g BW (DM + FETAA 40). The experiment was carried out for 21 days, at the end of the experimental period, the mice were then decapitated.

The fasting blood glucose assay

The fasting blood glucose concentration in serum was estimated with the GOD-PAP method using a glucose kit (DiaSys, Germany), and measured by spectrometry (SP-300, OPTIMA) with 500 nm wavelength. Blood samples were collected by retro-orbital vein, the experiment was carried out according to the manufacture's instruction.

The insulin level assay

Blood serum of mice was taken at the end of the experiment, analysis of insulin levels by Enzyme-Linked Immunosorbent Assay (ELISA) method using Mouse INS (Insulin) kit (Finetest, Wuhan) experiment was carried out according to the manufacturer's instruction.

The homeostasis model assessment for beta-cell function (HOMA-β)

The homeostasis model assessment for β-cell function (HOMA-β) was calculated via the following equation: fasting serum insulin (mU/L) x 20 / fasting glucose (mmol/L) - 3,5 [13]. The calculation used the HOMA2 Calculator software obtained from the Diabetes Trial Unit (DTU), University of Oxford 2004-2019.

The mRNA Pdx1 expression analysis with the Real-Time PCR

Total RNA was isolated from the pancreas for cDNA synthesis by using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The real-time PCR assay was performed by using ExcelTaq™ 2X Fast Q-PCR Master Mix (SMOBIO, China). Primers used were as follows: Gapdh forward 5'-CGTGCGTGACATCAAAGAGAA-3', reverse 5'-TGGATGCCACAGGATTCCAT-3'; Pdx-1 forward 5'- GAGGTGCTTACACAGCGGAA -3', reverse: 5'- GGGCCGGGAGATGTATTTGT -3'. The thermal cycling profile was used (40 cycles): 95 °C for 20 s, 95 °C for 3 s, 60 °C for 30 s.

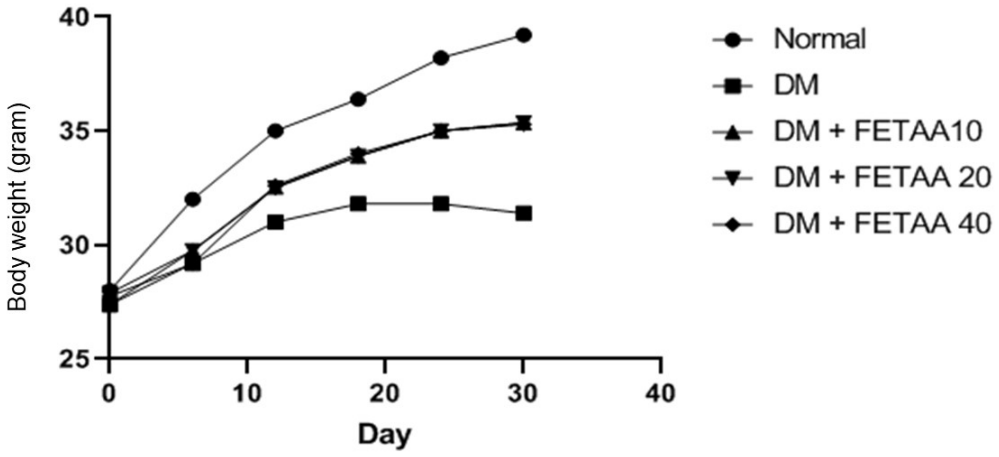

Figure 2. Curve of weight gain during experiment.

The $^{\Delta\Delta}$CT relative quantification method was used to determine the fold change in expression by normalizing the CT values of the target mRNAs to the CT values of the internal control Gapdh in the same samples. Further, it was normalized with the control ($^{\Delta\Delta}CT = {}^{\Delta}CT_{Treatment} - {}^{\Delta}CT_{Control}$). The fold change in expression was then obtained as $2^{-\Delta\Delta CT}$.

Data analysis

The data were drawn with Graphpad Prism 8.0 (San Diego, California, USA) and were expressed as the mean ± standard deviation. Statistical significance was analyzed by one-way analysis of variance followed Games Howell test. $p<0.05$ was considered to indicate a statistically significant difference.

Results

Determination of blood glucose and measurement of body weight

The fasting blood glucose levels were elevated in mice on a 5-day post administrated with STZ/NA. In this study, STZ/NA-treated mice that exhibited a fasting blood glucose level of ≥ 196 mg/dL were used in the study. The fasting blood glucose concentration in diabetic mice was significantly higher than in the diabetic group that received FETAA supplementation during the 3 weeks. After 3 weeks, the fasting blood glucose levels on DM + FETAA 10 group, DM + FETAA 20 group, and DM + FETAA 40 group were significantly lower than DM group ($p<0.05$) (Figure 1). Results showed that the administration of FETAA in each treatment group could reduce blood glucose levels in mice. The administration of FETAA 40 mg/100 g BW was able to reduce blood glucose levels higher compared to the other group. We also noticed that the overall group has gained body weight (Figure 2).

Effect of FETAA on insulin level

Insulin is a hormone that plays important role in decreasing blood glucose to maintain normal blood glucose concentration [14]. In order to know the effect of FETAA on insulin level, we carried out an insulin level assay using ELISA. We found that insulin level in the group that received FETAA was higher compared to the normal group. The group that received FETAA dose 20 mg/100 g BW has the highest insulin level. These data suggest that FETAA administration could increase insulin levels (Figure 3).

Effect of FETAA on HOMA-β Index

HOMA-β index is an equation derived from fasting glucose and insulin level. HOMA-β can be used to measure β cell function in the pancreas [15]. We found that HOMA β Index in DM + FETAA 10 group, DM + FETAA 20 group, and DM + FETAA 40 group were higher compared to the diabetic group. DM + FETAA 20 group showed significant

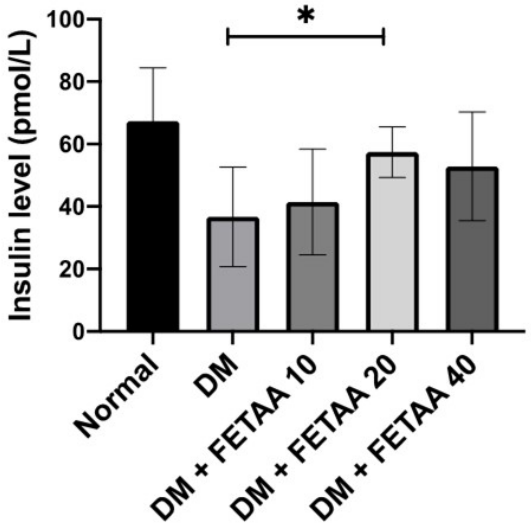

Figure 3. Insulin level. Statistical analysis using the One-way Anova test followed by the Howell Games post hoc test. *Express as significant (p<0.05)

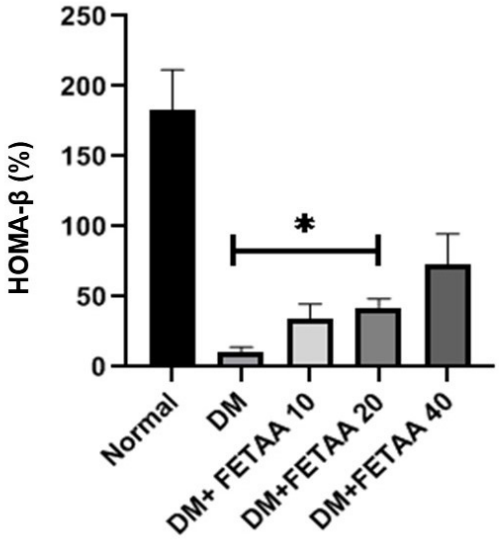

Figure 4. HOMA-β index. Data were analyzed with One Way Anova. * p<0.05

differences compared to the DM group. These data suggest that supplementation of FETAA could improve β-cell function (Figure 4).

Effect of FETAA on Pdx1 mRNA expression

Pdx1 was an important transcription factor that plays role in the development and function of the β-cell pancreas [16]. In order to know the effect of FETAA on Pdx1 mRNA expression, we carried out a Real-Time PCR analysis. We found that the FETAA-treatment group expressed a higher level of Pdx1 mRNA than the DM group, although there is no statistically significant (p>0,05) (Figure 5).

Discussion

In this study, the administration of fermented tempeh aerobic anaerobic (FETAA) could decrease blood glucose levels. This result agrees with another study that stated consumption of fermented soybean can reduce body weight and reduce blood sugar levels in obese Wistar rats [17]. The possible mechanism is that isoflavones and soy protein can increase the secretion of the hormone peptide YY (PYY) which functions to stimulate satiety and prevent eating in mice and humans. Isoflavones also could reduce fat accumulation in the body and increase insulin resistance. Isoflavones compounds in soybean fermentation products also reduce glucose production in the liver by increasing insulin signaling in the liver [18]. Daidzein and genistein are examples of isoflavone in soybean and soybeans products [19]. One of the mechanisms underlying was the increase in IRS2 phosphorylation, which increase the phosphorylation of AKT and reduce insulin resistance in the liver [20]. Administration of FETAA dose 20 mg/100 g BW showed an increase in insulin level compared to the normal group. This result is consistent with research that giving tempeh and soy fermented milk is known to increase insulin secretion and reduce blood glucose levels in diabetic rats induced with streptozotocin [21].

The data analysis of the HOMA-β index showed the group that received FETAA 20 mg/100 g BW had a higher HOMA-β index compared to the diabetic group. The result is consistent with another research that consumption of soy-based foods in pregnant women with diabetes mellitus, show improved glucose homeostasis parameters (HOMA-β and HOMA-IR), levels of oxidative stress, and the number of triglycerides [22]. In this study, HOMA-β Index was low compare to the normal group. In another study showed that measuring HOMA-β using insulin levels in serum is less representative of overall insulin levels due to

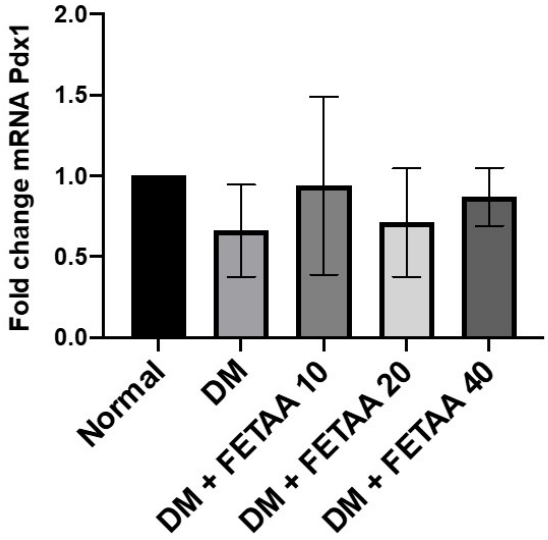

Figure 5. Fold change mRNA Pdx1. Statistical analysis using the One Way Anova test found no significant differences in each group (p=0.37)

the pattern of insulin secretion and short insulin half-life [23].

FETAA supplementation did not affect Pdx1 mRNA expression. Pdx1 is a transcription factor that plays important role in β-cell expansion and proliferation, which decreases the expression of Pdx1 expression could induce hyperglycemia [24]. Decrease of Pdx1 expression is known to be associated with decreased insulin content of β-cell and increase the susceptibility apoptosis of β-cell [25]. Other studies showed different results, the amino acid vglycin isolate from soybeans could increase the expression of the *Pdx1* gene which plays a role in the proliferation of pancreatic β-cells [26], as well as other studies regarding the administration of chitosan nanoparticle-GABA in diabetic mice, it can increase *Pdx1* gene expression compared with the diabetes group [27].

In this study, FETAA administration did not affect Pdx1 mRNA expression, although insulin levels in the group that received FETAA were higher than in the diabetic group. A possible mechanism according to a literature study regarding *Psamommys obesus* as a model of type 2 DM, suggested that the loss or reduced expression of the *Pdx1* gene can be compensated by other transcription factors to stabilize insulin gene expression and to maintain insulin levels in the pancreas [28]. Another mechanism showed that Pdx1 expression does not directly affect β-cell mass and function [29]. In contrast to other studies on GABA which promotes β-cell proliferation in diabetic mice using 6 mg/mL GABA [30]. In the present study, several parameters such as insulin levels, HOMA-β index, and Pdx1 mRNA expression showed an increase but were still not close to normal parameter values. The possible mechanism because the optimal dose of FETAA was not known. The limitation of this study is that it did not analyze the levels of active substances such as GABA and isoflavones in FETAA. It is necessary to develop other research using FETAA with different doses and different parameters.

Conclusion

Fermented tempeh aerobic anaerobic (FETAA) could improve HOMA-β index, lower blood glucose levels but did not affect Pdx1 mRNA expression.

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Author contribution

Conceptualization, H.W., P.; Methodology, H.W., P., P.H.; Investigation, H.W., P., P.H., A.H.S.; Resources, H.W., P.; Writing – Original Draft, H.W., P., P.H.; Writing – Review & Editing, H.W., P.; Supervision, P.

Decalaration of interest

The authors declare no conflict of interest.

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