Original Article

Tempeh-Based Supplement Decreases Blood Glucose Levels Through Inhibiting Rage and NF-κB Activity in Type 2 Diabetes Mellitus Mice Model

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Abstract:

Objective: Hyperglycemia promotes inflammation through inducing the formation of AGE products, which bind with receptor AGE (RAGE) products in cell membranes, leading to the activation of necrosis factor–kappa beta (NF-κB). This study aimed to analyze the effects of tempeh–based supplement (TBS) preparations of γ-amino butyric acid (GABA) tempeh against mRNA expressions of RAGE and NF-κB on the pancreas of a type 2 diabetes mellitus (DM) mice model.

Material and Methods: This research was a quasi–experiment, with pre and post–tests with a control design for blood glucose levels; and post–test only utilizing control for mRNA RAGE and NF-κB expressions. A total of 30 male mice, 8–10 weeks old, weighing 20–25 g were divided into 6 treatment groups: non–diabetic, Diabetic, Diabetic+metformin, Diabetic+TBS 10 mg/100 g BW, Diabetic+TBS 20 mg/100 g BW, and Diabetic+TBS 40 mg/100 g BW. STZ induction once a day for two days was preceded by NA to create a DM mice model; meanwhile, TBS was administrated once a day for 21 days.

Results: The mean difference of fasting glucose levels in the diabetic+TBS 40 mg/100 g BW group was the highest when compared to the diabetic group (159.52±1.85) mg/dL. One-way ANOVA revealed statistically significant differences in fasting glucose levels, RAGE and NF-κB expressions in the Diabetic+TBS group at various dosage levels compared

to the diabetic control group. Relative mRNA expressions of RAGE and NF-κB were downregulated in the treatment group compared to the diabetic control group.

Conclusion: TBS can decrease fasting blood glucose levels and downregulate relative mRNA expressions of RAGE and NF-κB in type 2 DM mice.

Keywords: diabetes mellitus, NF-κB, RAGE, TBS, tempeh

Introduction

Diabetes mellitus (DM) has become an important global health problem. The World Health Organization (WHO) reported that the prevalence of DM had increased from 4.7% in 1980 to 8.5% in 2014; globally[1]. Worldwide, more than 425 million people are influenced by DM and its comorbidities, which include diabetic neuropathy, nephropathy, retinopathy and other serious complications[2-4]. Riskesdas 2018, reported that the prevalence of DM in Indonesia increased from 6.9% in 2013 to 8.9% in 2018 (Ministry of Health, 2018). Type 2 DM is a major concern because it covers 90% of all patients with diabetes in the community[5].

Chronic inflammation has an important role in the pathogenesis of type 2 DM as it promotes a decrease in insulin secretion and sensitivity, leading to a diminishing of pancreatic mass. Inflammation also plays a role in the pathogenesis of diabetic complications; such as vascular dysfunction, nephropathy, neuropathy and retinopathy[6]. Additionally, it promotes the increasing formation of advanced glycation end (AGE) products, which when bound to advanced glycation end (RAGE) products' receptors in the cell membranes. This then leads to necrosis factor–kappa beta (NF-κB) activation[7]. NF-κB is a transcription factor found in all cell types that is responsible for transcriptional of various proteins, which includes pro-inflammatory and inflammatory molecules; such as cytokines (eg. TNF-α), chemokines (eg. MCP-1), cell adhesion molecules (CAM) (eg. vascular cell adhesion molecule-1 (VCAM-1)) free radicals and different enzymes (eg. iNOS and COX-2). The presence of these proteins plays an important role in the pathophysiology of diabetes as well as its micro- and macrovascular complications[8-11].

Tempeh is a traditional Indonesian food made from soybean, fermented with *Rhizopus Oligosporus.* It has several bioactive components that are useful for reducing inflammation; such as isoflavones, antioxidants and γ-amino butyric acid (GABA). Previous studies revealed that isoflavones and GABA can induce insulin secretion[12,13]. Genistein (isoflavone derivative in tempeh-based supplement (TBS)) and GABA can inhibit NF-κB through ERK, Jak/STAT and NADPH oxidase pathways, respectively[14]. Additionally, research further showed that GABA can induce the survival of pancreatic beta cells[13]. TBS has several antioxidant constituents that can ameliorate inflammation[15]; these being: 3-hydroxyanthranilic acid HAA, amino acids, peptides and phenol compounds. All of which can inactivate ROS generated by the NADPH oxidase pathway, leading to inhibition of NF-κB activation[14,16].

Several benefits of modified tempeh have been studied; such as its hepatoprotective[17], neuroprotective[18] and antihypertensive effects[19]. Although, the benefits of tempeh in DM have been widely studied, it still remains unclear whether modified tempeh has an effect on inflammation in the pancreas. Hence, this study aimed to determine the effects of TBS against the expressions of RAGE and NF-κB in a type 2 DM mice model.

Material and Methods

The preparation of TBS

The TBS formulation was made via several steps, including: boiling, soaking, peeling the skin, washing, draining, inoculating, wrapping and fermenting[20]. Then, the aerobic fermentation process for 30 hours continued, with anaerobic fermentation for 20 hours[19,21,22]. Finally, the TBS was made into flour by freeze-drying it for 3 days.

Animal subjects

Ethical approval for the experimental use of test animals was granted by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing University Gadjah Mada. As many as 30 normal, healthy mice (male) were selected: aged between 8-10 weeks old, with a range of body weight from 20-25 g. They were kept in cages in a 12-hour day and 12-hour night cycle. The temperature was maintained at 22-25 °C[23]. Prior to the commencement of experiments, acclimatization of the animals was undertaken for 7 days. An AIN-93M diet was provided to the animals, with fresh drinking water ad libitum.

Experimental design

This study used intraperitoneal injection of STZ/NA to make the type 2 DM mice model: referring to the study of[24,25]. Nicotinamide 120 mg/kg body weight (BW) dissolved in saline was injected peritoneally 15 minutes before Streptozotocin (STZ) 50 mg/kg BW in 0.1 M citrate buffer, with pH 4.2 being injected peritoneally once a day for two days. Blood was collected by the retro-orbital puncture method for measuring fasting blood glucose levels, using glucose oxidase and peroxidase kit methods with spectrophotometry. Animals with a fasting blood glucose (FBG) concentration of 200 mg/dl or more were considered as type 2 DM in this experiment[26].

Mice were grouped into 6 groups (n=5).

Group I – Nondiabetic control

Group II – Diabetic control

Group III – Diabetic+metformin 2.6 mg/day

Group IV – Diabetic+TBS 10 mg/100 g BW

Group V – Diabetic+TBS 20 mg/100 g BW

Group VI – Diabetic+TBS 40 mg/100 g BW

All mice underwent treatment once a day for 21 days; as mentioned above. Blood glucose levels of the fasting animals of group I–VI were determined on day 0 and 21.

Gene expression assay

Harvesting of mice were conduct by disacrifice and pancreas tissue stored in a –80 °C. mRNA was extracted from pancreatic tissue using TriRNA reagent. The purity of mRNA concentrations was measured with a NanoDrop spectrophotometer. The extracted mRNA was made into cDNA using reverse transcriptase-polymerase chain reaction (RT-PCR). The GAPDH gene was used as the house-keeping gene for normalization. Realtime RT-PCR was perfromed according to procedures in qPCR kit Excel Taq (Smobio Hsinchu City, Taiwan). The primers were used for quantitative PCR, with sequence RAGE F:5'-CGGGACTCTTTACACTGCGG-3'R:5'-GGAGAAGGTAGGATGGGTGGTT-3', NF-κB F:5'TCCGTCTGTCTGCTCTCTCT-3'R:5'CTGTCATCCGTGCTTCCAGT-3' and reference gene primer for GAPDH F:5'GGTCCCAGCTTAGGTTCATCA-3'R:5'-ATCCGTTCACACCGACCTTC-3'. The running were conducted at settings: (1) Initial denaturation: 95 °C for 10 min; (2) denaturation: 95 °C for 15 s; (3) annealing: 57 °C for 1 min; (4) elongation: 72 °C for 30 seconds. Relative expressions of mRNA were calculated by the livak method ($2^{-\Delta\Delta CT}$).

Statistical analysis

Blood glucose levels and mRNA expression are presented by mean and standard deviation (S.D.). The data were then tested for their normal distribution using the Saphiro-Wilk test. One-way ANOVA analysis was performed within the groups of data, followed by post hoc tests. p-value<0.05 were considered as significant differences.

Results

Blood glucose level

Biochemical assay results of the blood glucose levels from each TBS group were statistically significant compared to the negative control group (Figure 1). Figure 2 shows the decrease in blood glucose levels; except in the diabetic control group. The reduction in FBG in the treatment groups was statistically significant, with a p-value<0.05 compared to the diabetes control group. This result shows that giving TBS can significantly reduce fasting blood sugar levels in mice (p-value<0.001).

Gene expression analysis in each group

RT-PCR was performed on the pancreas of each group, with primers corresponding to RAGE and one housekeeping gene, GADPH, to support biochemical changes in our observations. Figure 3 shows the RT-PCR amplified product being significant in the diabetic+TBS 10 and 40 mg/100 g groups compared to the diabetic control group. This finding indicated that the reduction of relative expression of RAGE mRNA occurred in the diabetic group, which had been administered between 10 and 40 mg/g body weight of TBS.

Figure 3 shows that there was a significant difference between the mean expression of the NF-κB gene in the treatment group and the diabetic group. This finding indicated that the reduction of relative expression of NF-κB mRNA occurred in the diabetic group, which had been administered between 10 and 20 mg/100gBW of TBS.
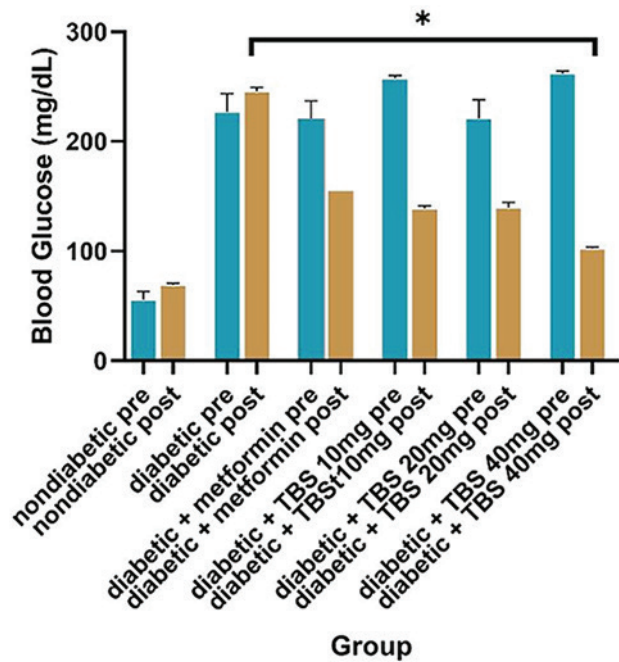

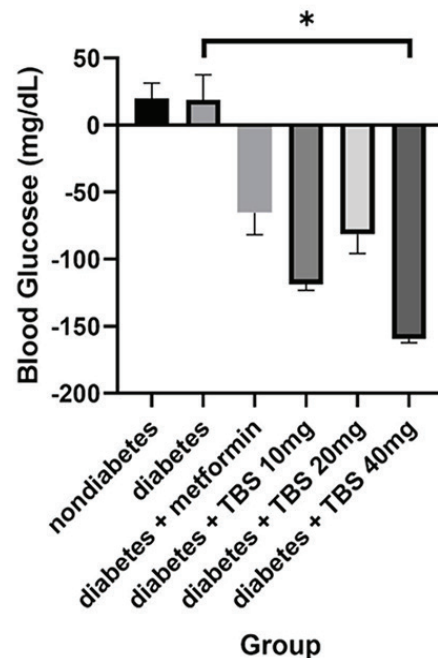**Figure 1** Blood glucose level pre and post-treatment with tempeh-based supplement (TBS)

Figure 2 Decrease of blood glucose level after treatment with tempeh-based supplement (TBS)
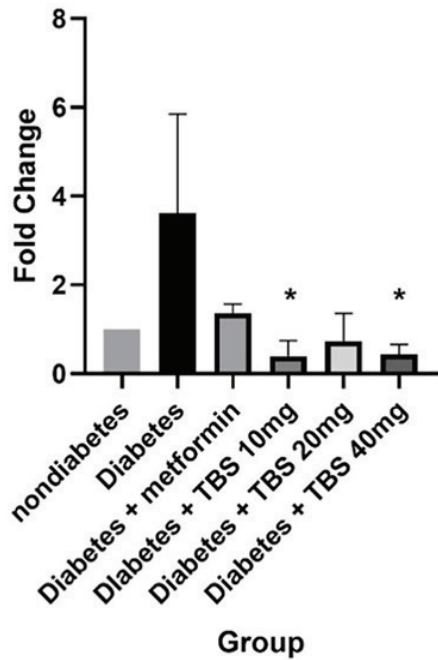
Figure 3 Relative mRNA of RAGE e7xpression after treatment with tempeh–based supplement (TBS)
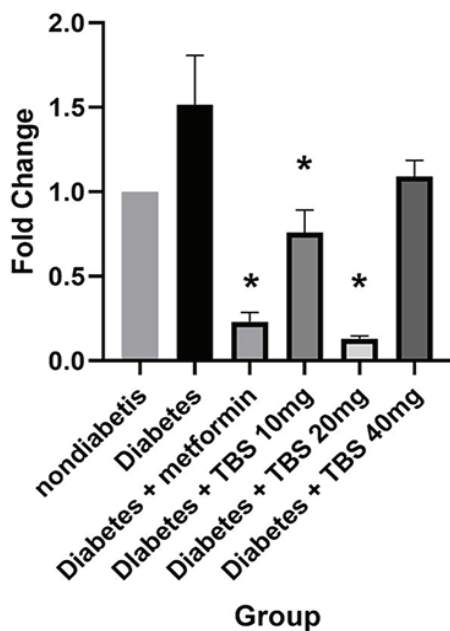

Figure 4 Relative mRNA of NF–kB expression after treatment with Tempeh–based supplement (TBS)

Discussion

This study showed a significant decrease in blood glucose between the groups treated with TBS compared to the diabetic group. The duration of treatment for 21 days supports an increase in the amount of probiotics *A. muciniphilia;* based on the study conducted by Stephanie *et al.,* which reported that an intake of TBS can increase the bacteria, *Akkermansia muciniphila* (*A. muciniphilia*) up to 35 times after 28 days; if consumed regularly. This is associated with lower blood glucose levels, higher insulin sensitivity, and increased endocannabinoid levels that can control inflammation, gut defenses and gut peptide secretion. Additionally, it is associated with decreased levels of blood glucose, and inflammatory factors; such as NF–kB[27]. TBS that has undergone a fermentation process will have different nutritional components from soybeans because there are bioactive changes that make the nutritional components more easily absorbed; for example, in oligosaccharides and isoflavones changing from glycosides to aglycones[28,29]. Stephanie et al. (2019) reported that there were increases of several bacteria; such as *Bifidobacterium* spp. and *A. muciniphila,* after consuming TBS. *A. Muciniphilia* had an influence on insulin secretion and sensitivity, could lower blood glucose levels and helped establish a good metabolic index[27,30]. The probiotic can secrete propionate, a compound that can stimulate secretion of glucagon–like peptide–1 (GLP–1) to increase insulin secretion and appetite suppression. This compound can also modulate the expression of peroxisome proliferator–activated receptor gamma (PPARγ), a protein that plays an important role in glucose homestasis and increased insulin sensitivity[31,32]. Additionally, *A. muciniphila* can also improve insulin sensitivity and glucose tolerance by inhibiting the JNK signaling pathway and increasing expression of inhibitor NF–κB levels in the liver[33]. On another side, in this research, metformin was used as the

positive control as control, as it has been used as a first–line therapy for type 2 diabetes for over half a decade. It acts as an AMPK activator that leads to inhibition of an AGEs–induced inflammatory response as well as suppressing the RAGE∕NF–κB signaling pathway[34,35].

Recently the process of formulation of TBS has attracted the attention of several researchers; for example, adding an anaerobic fermentation process after aerobic fermentation. This can increase the components of GABA, free amino acids, and oligopeptides. Furthermore, the pH of TBS became acidic, which is an optimal pH to produce GABA by glutamate decarboxylase[21]. Other studies have shown that GABA components can act as antidiabetic agents. GABA can stimulate insulin release by pancreatic beta cells[13]. In addition, GABA also improves insulin resistance in mice with high fat diets by increasing the activation of Treg cells to suppress inflammation[36].

In addition to these benefits, the fermentation TBS process also makes the TBS isoflavones' content higher than other soy products. Genistein is a one of the isoflavones that plays an important role. It is also the most abundant isoflavone in TBS, which can protect pancreatic beta cells against cytokines through inhibition of inducible nitic oxide synthase (iNOS) and nitric oxide gene expression, suppression of extracellular signal–regulated protein kinases 1 and 2 (ERK1∕2) and Jak∕signal transducer and activator of transcription (STAT)[15]. Genistein can also inhibit several inflammatory cytokines; including tumor necrosis factor–alpha (TNF–α) and Interluekin–6 (IL–6) as well as the degradation of NF–κB and components of p50, p65 and Ikβ in the cytosol[37]. The decreasing of fasting blood glucose levels of mice treated with TBS was also a result of an increase in insulin secretion through increased glucose stimulation by genistein. Additionally, there was an increase in cAMP (through increased adenylate cyclase activity), which promotes the PKA pathway and leads to insulin secretion[12]. This mechanism can ameliorate hyperglycemia[38].

The relative expression of RAGE gene in the TBS treated groups was downregulated compared to the diabetic control group. This finding was in line with the decreasing of the FBG levels in the mice. This study revealed a statistically significant decrease in FBG levels in the groups treated by TBS compared to the diabetic control group. However, the diabetic+TBS 20 mg group did not show a statically significant decreasing of relative expression RAGE gene compared to the diabetic control group. Also, it was not significantly different from the positive control group, diabetic+metformin group. In this study, it was also found that the diabetes group with 20 mg TBS had higher RAGE mRNA expression than the other two doses. This probably happened because the average decrease in blood glucose in the 20 mg TBS was the lowest compared to other groups, in that blood glucose levels were higher than in other groups. RAGE formation depends on blood glucose levels, so that the 20 mg group has the highest RAGE compared to other groups.

One study reported that genistein in TBS can reduce serum methylglyoxal, which is one of the reactive glucoses that will bind with protein to form AGE. In addition, genistein can also increase GSH in the serum, which can neutralize methylglyoxal[38]. The main role of antioxidants is to neutralize methylglyoxal and free radicals. One of the signaling pathways triggered by the AGE–RAGE interaction is NADPH–oxidase. When active, NADPH–oxidase produces superoxide anions (O^{2-}). Excessive free radicals will increase the production of AGE and AGE–RAGE bonds, and AGE–RAGE will activate the NADPH–oxidase pathway leading to NF–κB activation, which increases the production of free radicals and then the process is repeated[7]. Normally, the body needs a few free radicals to promote the immune system. In addition, ROS plays an important role in mediating the transduction of the RAGE signal[39,40].

The reduction in the relative expression of NF-κB was significantly different between the diabetic+TBS 10 mg/100 g BW and 20 mg/100 g BW groups. Meanwhile, a dose of 40 mg/100 g BW was not significantly different from the diabetic control group. This explains that 20 mg/100 g BW is suggested as the maximum dose that can reduce NF-κB levels, and increasing this does not increase the effect on gene expression. Additionally, the decrease in glucose levels after administration of 40 mg TBS was the highest. However, NF-κB mRNA expression was the highest among the group treated with metformin and TBS, this was higher than in the non-diabetic group: possibly this occurred through other NF-κB activation pathways that have not been explored in this study.

This is assumed to be due to the potency of the genistein and GABA components, which can inhibit NF-κB activation. Inhibition of NF-κB activation by genistein and GABA occurs through the ERK, Jak/STAT pathways and p65 deacetylation, respectively. Additionally, genistein inhibits the expression of inducible nitric oxide synthase genes (iNOS) and nitric oxide production, which will promote degradation of NF-κB and its components be (p50, p65, and Ikβ) in the cytosol[15]. Some inflammatory cytokines that are inhibited by genistein include TNF-α and IL-6[37]. GABA also plays a role in activating the expression of SIRT1 protein that can deactivate NF-κB, and AKT through influx Ca^{2+}, leading to increased insulin secretion. Inactivation of NF-κB is important because in pancreatic beta cells NF-κB triggers inflammation, which can lead to autoimmune activation and insulin resistance in patients with type 2 DM. Antioxidants in TBS can also deactivate free radicals, including ROS produced by the NADPH oxidase pathway[41]. As explained, NF-κB plays an active role in the pathogenesis of DM. In type 2 DM the activation of NF-κB causes an inflammatory reaction leading to insulin resistance.

There are several limitations in this study, such as the treatment time for 21 days, which was considered less than optimal to trigger RAGE and NF-κB gene expressions. Measurement of food residue was also not done. Further research is needed to confirm this study's findings.

Conclusion

The results of the present investigation suggested the administration of a tempeh based supplement can decrease fasting blood glucose, down-regulate RAGE and NF-κB expressions and can be considered as a natural food agent that controls diabetic complications.

Ethical approval of research

The Ethical Approval of this research comes from The Ethical Committee of Research in Medical Health, Faculty of Medicine, Public Health, and Nursing; reference: KE/0195/02/2019.

Acknowledgement

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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