Basic Medical Research

Soybean extract increases telomerase reverse transcriptase protein expression in pancreatic β-cells of diabetes mellitus-induced rats

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ABSTRACT

BACKGROUND A number of studies on the benefits of soybean (*Glycine max* (L.) Merr) in the treatment of diabetes mellitus (DM) have already been conducted; however, the effects of soybean extracts on telomerase reverse transcriptase (TERT) expression in improving telomerase activity in pancreatic cells is currently unknown. This study was aimed to evaluate the effects of soybean extracts on TERT protein expression in the pancreatic β-cells of rats with DM.

METHODS Sixty male Sprague-Dawley rats were randomly divided into six groups: (1) negative control (N); (2) DM rats induced by alloxan (DMA); (3) DM rats with glibenclamide (DMG; positive control); (4) DM rats with 1 mg/kgBW/day soybean extract (DM1E); (5) DM rats with 5 mg/kgBW/day soybean extract (DM5E); (6) DM rats with 25 mg/kgBW/day soybean extract (DM25E). The treatments were carried out over 28 days. The measured variables included fasting blood glucose (FBG) level, TERT protein expression, and the number of pancreatic β-cells.

RESULTS All parameters were measured against the diabetes control group. The FBG levels in rats DM1E, DM5E, and DM25E were significantly reduced on the 28th day ($p < 0.05$). TERT protein expression and the number of pancreatic β-cells (DM25E) also showed significant improvements compared to DM rats ($p < 0.05$).

CONCLUSIONS Soybean extracts can increase TERT protein expression in pancreatic β-cells in diabetes-induced rats.

KEYWORDS diabetes mellitus, rats, soybeans, telomerase reverse transcriptase

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting in increased free radical production, particularly in reactive oxygen species (ROS).[1] An imbalance between ROS production and protective cellular mechanisms results in damage to DNA, cellular dysfunction, and tissue injury, which are the main complications of DM.[2,3] Chronic hyperglycemia reduces β-cell numbers as reported by Zhang et al,[4] who demonstrated a 3–10 fold increase in β-cells apoptosis in pancreatic autopsy of patients with type-2 DM compared to individuals without diabetes. Some studies suggested that the telomere length of type-2 DM patients was significantly shorter than the non-DM control was ascribed to higher oxidative stress under diabetic conditions.[5,6] The telomere is a DNA structure located at the terminal of all chromosomes. Telomere DNA is synthesized and maintained by telomerase enzyme.[7]

Telomerase consists of two essential subunits; that is, telomerase reverse transcriptase (TERT) enzyme and the telomerase RNA component (TERC

or TR). The activity of telomerase is determined from the expressions of two major genes (i.e., TERT and TERC). TERT encodes reverse transcriptase and TERC encodes RNA template. These genes are considered to be among the most important genes for enzyme functioning, since increased telomerase activity is highly correlated to the amount of TERT mRNA.[8] Disturbance of TERT or TERC can result in either reduced or increased telomerase activity. Kuhlow et al[6] reported that in DM rats without TERC, telomerase activity was reduced and telomere shortening occurred more rapidly. Increased cellular ROS causes TERT to flow out of the nucleus and into the cytoplasm.[9] In order to prevent DNA damage, increasing the activity of telomerase can reduce the rate of telomere shortening, cell dysfunction, and tissue injury. Antioxidants are necessary to reduce the oxidative stress.

Soybean (*Glycine max* [L.] Merr) contains a high amount of genistein, an antioxidant that can reduce ROS production. Moreover, it can alleviate some of the other symptoms of diabetes.[10] *In vitro* studies on genistein (5–10 μmol/l) have shown direct effects on pancreatic β-cells. The antioxidant stimulates insulin secretion by activating the cAMP-dependent protein kinase (PKA).[11] Mustofa et al[12] also reported that supplementation of soybean porridge at a dose of 500 mg per kg of body weight per day (kgBW/day) for 4 weeks in diabetic rats can cause increased expression of the insulin gene upon histopathology of pancreatic β-cells.

Based on the results of these findings, this study investigate whether soy extract treatments could change insulin gene and TERT protein expressions in the pancreatic β-cells of diabetes-induced rats.

METHODS

This study was approved by the Ethics Committee of Faculty of Medicine, Universitas Indonesia, no.118/H2/F1/ETIK/2013.

Experimental animal model

Sixty healthy 10–12 weeks old male Sprague-Dawley rats (*Rattus novergicus*), (weight of 200–250 g), were obtained from the Laboratory of Nutrition, Faculty of Medicine, Universitas Indonesia, Jakarta. Prior to the study, the rats were acclimatized to the cages for a week at a room temperature (27°C) with

an adequate air ventilation and a standard diet. After acclimatization, the rats fasted (no food or drink) for 16–18 hours. On the 8th day, their blood glucose levels were measured using a glucometer (Accu-Chek®), and the rats were divided into six treatment groups, with 10 replicate rats in each group. The treatment groups were: (1) negative control (N), (2) DM rats induced by alloxan (DMA), (3) DM rats with 5 mg glibenclamide (DMG; positive control), (4) DM rats with 1 mg/kgBW/day soybean extract (DM1E), (5) DM rats with 5 mg/kgBW/day soybean extracts (DM5E), and (6) DM rats with soybean extracts 25 mg/kgBW/day (DM25E). Soybean extracts were diluted in 1% Carboxymethyl cellulose (CMC) and were given to the rats *ad libitum.* On the 28th day, the rats were anesthetized with ether then killed by decapitation, and their pancreas was analyzed using histochemical examination.

Soybean extracts

Soybean (*Glycine max* [L.] Merr) extracts were obtained by maceration. The extraction conducted at the Biopharmaceutical Research Center of Institut Pertanian Bogor. About 100 g of soybeans was blended into porridge and was subsequently macerated in 250 ml 70% ethanol for 24 hours. The samples were strained and the filtrates were collected. The residual samples were added with 100 ml 70% ethanol and macerated for 24 hours. Later, the samples were strained again and the filtrates were collected. The second residual sample was added to 100 ml 70% ethanol. The filtrates were then concentrated using a rotary evaporator until a thick extract was produced. The extract was then put into an oven at 50°C for 30 min and an isolate was obtained. The doses used were 1 mg/kgBW, 5 mg/kgBW, and 25 mg/kgBW, all given to rats in 1% CMC solution once daily over 28 days orally.

Induction of diabetes

DM was induced in rats by intraperitoneal injection of 150 mg/kgBW alloxan monohydrate (Sigma, St. Louis, MO, USA) diluted in 0.9% sterile saline solution. Prior to the injection, the rats were fasted for 18 hours.[13] After 72 hours (3 days), blood samples were drawn from tail tip of rats to evaluate their plasma glucose level using a Accu-Chek® glucometer (Roche, Germany). Fifty rats with higher fasting blood glucose (FBG) levels (≥200 mg/dl)

were included as subjects (normal FBG level in rats is 85–100 mg/dl).[14] Normal non-DM rats were injected with 2 ml citrate buffer. Blood glucose levels were measured on the 3rd, 14th, and 28th days following the alloxan injection.

Immunohistochemistry staining

Immunohistochemistry (IHC) staining was performed at the Laboratory of Histology, Universitas Indonesia to observe the distribution of insulin-producing β-cells of the islets of Langerhans. The first step was deparaffinization and rehydration for 30 min. Deparaffinization involved eliminating the paraffin in which the specimens were immersed through a series of solutions (i.e., xylol III, xylol II, and xylol I). Then, the rehydration process was carried out by immersing the specimens through a series of alcohol solutions (95%, 90%, 80%, and 70%), with 30 min in each solution. Afterwards, the specimens were washed using distilled water (DW) and rinsed in phosphate buffer saline (PBS) three times, and then were lined with a dakopen marker. Specimens were then incubated in normal serum for 30 min and rinsed with PBS solution 3 times, incubated with anti-TERT antibody (NB100-317, Novus Biologicals LLC) in a refrigerator overnight, and were rinsed again with PBS 3 times. The specimens were subsequently reacted with an Envision Kit for 30 min and rinsed with PBS solution 3 times. Lastly, they were visualized using 1,3-diaminobenzidine, rinsed with DW followed by dehydration, clearing, and mounting.

Image analysis

Histopathological observation of the pancreatic β-cells was performed in parts of the islets of Langerhans using hematoxylin and eosin and IHC staining with TERT antibody. The IHC staining was used to identify TERT protein expressions in the β-cells. The results of IHC staining on the islets of Langerhans were observed qualitatively using a light microscope under 400× and 100× magnification. Pancreatic cells brown in color (from IHC staining) were counted digitally to observe TERT protein expression. Counting was carried out under the microscope, followed by analysis using Image Raster software (Optilab LLC).

Differences in expression between positive and negative for telomerase could be observed from differences in the brown color in the cell nucleus. The telomerase was considered positive when there was its activity had a positive nucleus signal with an obvious expression of the TERT protein in pancreatic β-cells. While telomerase which had negative nucleus signal and the expression of TERT protein was not obvious on β-cells was considered negative.[15]

Cell count

The counting of cells with positive and negative expressions was carried out by distinguishing the different colors in the cell nucleus. The coloring was done using an Image Raster (Optilab). By counting cell nuclei with the same color, data on the number of telomerase cells positive (pink color) and telomerase cells negative (blue color) were obtained. The following parameters were recorded in this study: 1) Average number of pancreatic β-cells; 2) average number of pancreatic β-cells positive for telomerase expression; 3) average number of pancreatic β-cells negative for telomerase expression; and 4) the percentages (%) of pancreatic β-cells positive or negative for telomerase expression. The average number of pancreatic β-cells positive for telomerase within the same group was counted by adding up the pink colored cells of islets from the same group divided by the total number of Langerhans islets. Likewise, the average number of pancreatic β-cells negative for telomerase activity was also obtained. That is, by adding up all cells that were blue in color. The average number of pancreatic β-cells was determined by the sum of the average number of pancreatic β-cells positive for telomerase and those negative for telomerase. The mean percentage (%) of pancreatic β-cells positive for telomerase activity was calculated by dividing the average number of pancreatic β-cells positive for telomerase with the total average number of pancreatic β-cells multiplied by 100. Likewise, the mean percentage (%) of pancreatic β-cells with negative telomerase activity was also determined.

Data analysis

Data analysis was performed using SPSS software, version 20 (IBM). Normalization test was carried out using Kolmogorov–Smirnov test. Where the data had a normal distribution, one-way analysis of variance (ANOVA) test was conducted using a significance level of $p < 0.05$. When the data had a
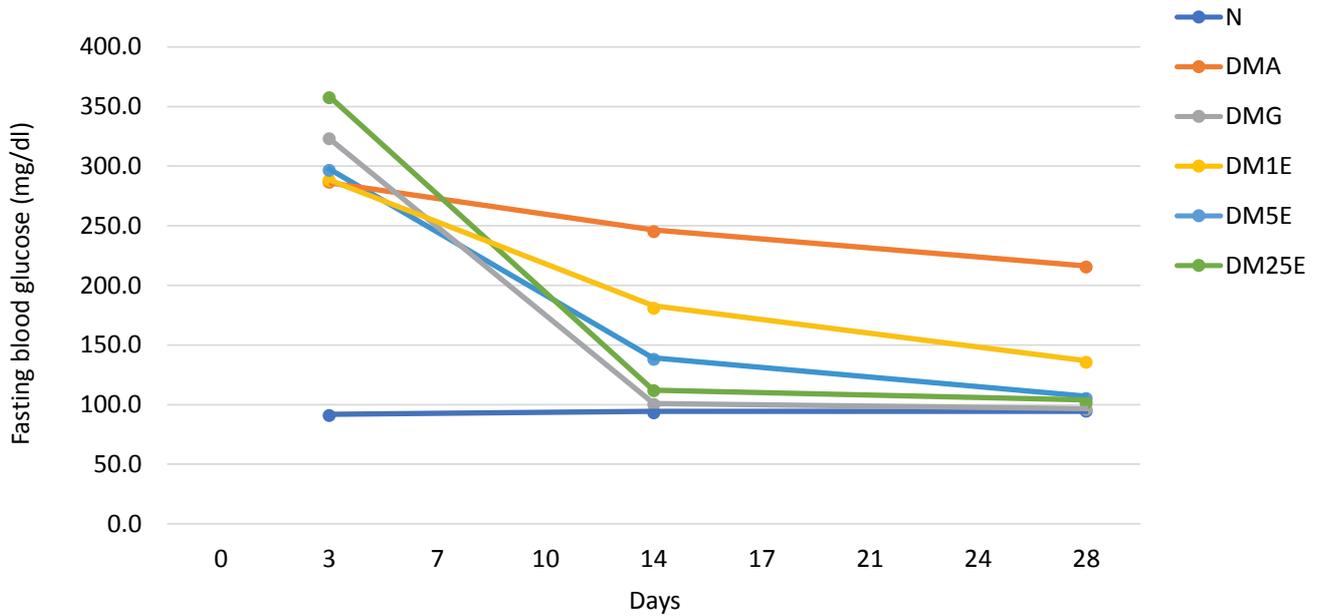
Figure 1. Changes in FBG levels in rats on the 3rd, 14th, and 28th day. On the 14th day, FBG levels in the negative control, DMG, DM5E, and DM25E were lower than DM control (DMA) ($p < 0.05$). *$p < 0.05$ in rats with DM. FBG=fasting blood glucose; N=normal rats; DMA=DM rats induced by alloxan, DMG=DM rats with glibenclamide; DM1E=DM rats with 1 mg/kgBW/day soybean extract; DM5E=DM rats with 5 mg/kgBW/day soybean extract; DM25E=DM rats with 25 mg/kgBW/day soybean extract; DM=diabetes mellitus

non-normal distribution, non-parametric tests were performed (i.e., Kruskal–Wallis test followed by Mann–Whitney test).

RESULTS

Fasting blood glucose (FBG) level

The diabetic rats had reduced FBG levels, but these levels were still greater than that of the non-DM rats. In rats from the DM with glibenclamide treatment group, there was a significant decrease of FBG level. The FBG level was reduced in DM rats across soybean extract treatment groups (i.e., 1 mg, 5 mg, and 25 mg/kgBW/day), as illustrated in Figure 1.

Telomerase expression on pancreatic β-cells: IHC staining

This qualitative study on telomerase expression of the islets of Langerhans found differences among non-DM rats, DM rats, and rats with soybean extract supplementation (1, 5, and 25 mg/kgBW/day). Figure 2 shows the histopathology of pancreatic islets with different telomerase expression. Figure 2a (non-DM rats) shows a more prominent TERT expression compared to that of Figure 2b. That is, rats with DM and rats of other treatment groups had different TERT

protein expressions in the nucleus and cytoplasm of β-cells in the islets of Langerhans. Figure 2b shows that there was less TERT protein expression in the islets compared to in the nucleus and cytoplasm. Figure 2c illustrates the DM with glibenclamide treatment group, displayed more TERT protein expression compared to that of Figure 2 (b, d, e, and f).

The average number of pancreatic β-cells

IHC staining produces different expression in the nucleus of cells. Therefore, it can be used to count the average number of cells. The average number of cells in non-DM rats was greater than that of other groups (mean [SD] = 94.9 [40.2]); while the average cell number for DM rats was (mean [SD] = 39.1 [24.2]). The groups of rats treated with soybean extract showed a greater number of cells compared to DM rats. The ANOVA showed a significant difference in the average number of pancreatic cells between non-DM rats and rats with DM ($p < 0.05$) (Figure 3).

The average number of pancreatic β-cells with positive telomerase

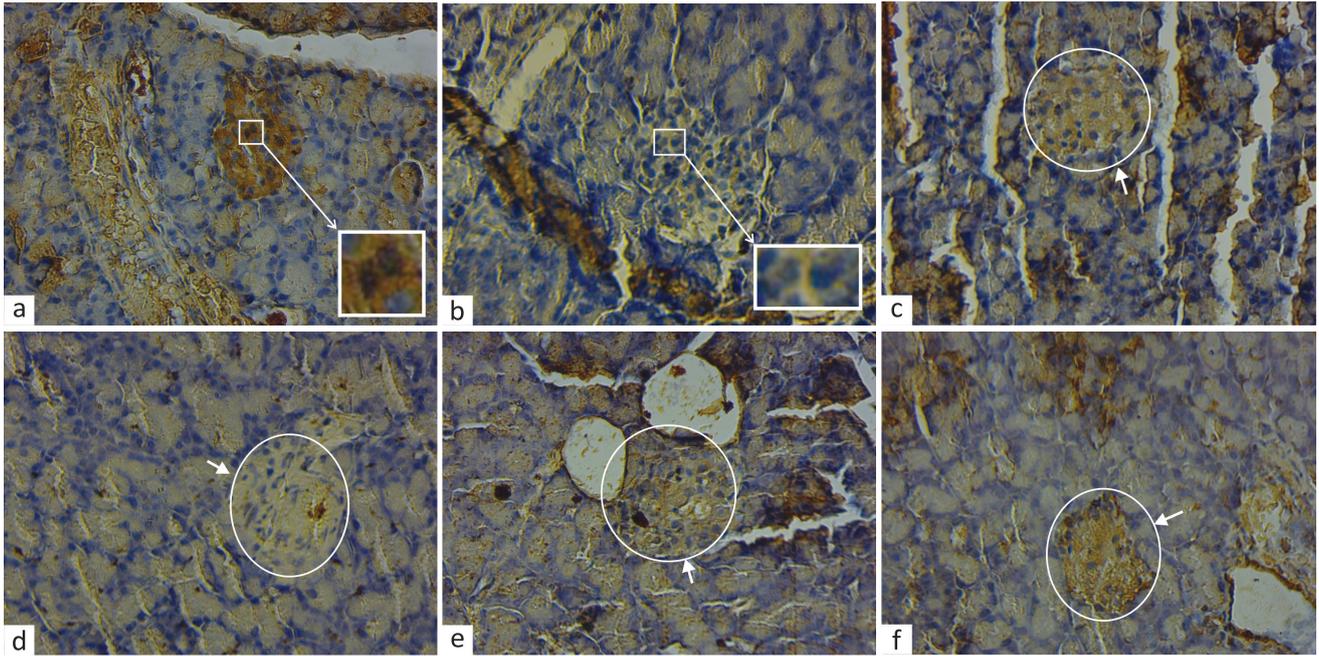DM rats had the lowest average number of positive telomerase cells (mean [SD] = 1.0 [2.1]).

Figure 2. Structure of rats pancreas on the 28th day with IHC staining (400 × 10 magnification, scale bar, 10 μm). The arrow shows cell expressions in the islets of Langerhans. (a) negative control; (b) DM control (DMA); (c) Positive control (DMG); (d) DM1E; (e) DM5E; and (f) DM25E. The cells with positive expressions (in the box figure a) and the cells with negative expressions (in the box figure b). IHC=immunohistochemical; DMA=DM rats induced by alloxan, DMG=DM rats with glibenclamide; DM1E=DM rats with 1 mg/kgBW/day soybean extract; DM5E=DM rats with 5 mg/kgBW/day soybean extract; DM25E=DM rats with 25 mg/kgBW/day soybean extract; TERT=telomerase reverse transcriptase
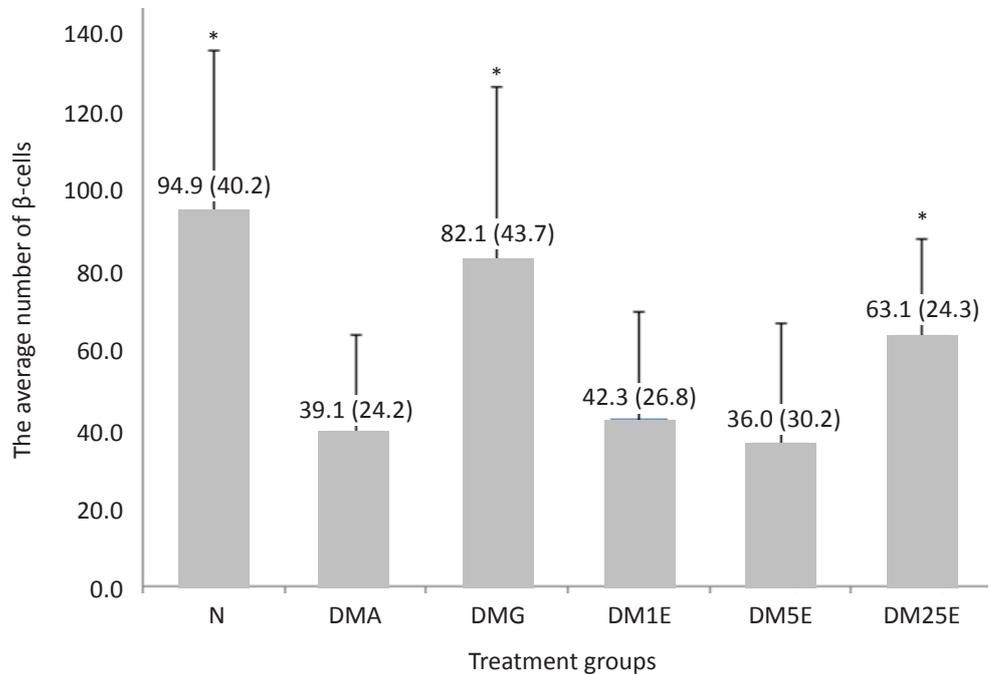
Figure 3. The average number of pancreatic β-cells on the 28th day. Asterisks indicate significant differences ($p < 0.05$) between each group compared to DMA group. N=normal rats; DMA=DM rats induced by alloxan, DMG=DM rats with glibenclamide; DM1E=DM rats with 1 mg/kgBW/day soybean extract; DM5E=DM rats with 5 mg/kgBW/day soybean extract; DM25E=DM rats with 25 mg/kgBW/day soybean extract

In contrast, non-DM rats had the highest average number of positive telomerase cells (92.0 [38.3]). ANOVA showed a significant difference in the average number of pancreatic β-cells with positive telomerase between non-DM rats and rats with DM ($p < 0.05$) (Figure 4).

DISCUSSION

This study shows that the supplementation of soy extracts can reduce blood glucose levels in diabetic rats. It is assumed that isoflavone genistein reduces the blood glucose level of rats through

Figure 4. The number of pancreatic β-cells with positive telomerase on the 28th day. Asterisks indicate significant differences ($p < 0.05$) between each group compared to DMA group. N=normal rats; DMA=DM rats induced by alloxan, DMG=DM rats with glibenclamide; DM1E=DM rats with 1 mg/kgBW/day soybean extract; DM5E=DM rats with 5 mg/kgBW/day soybean extract; DM25E=DM rats with 25 mg/kgBW/day soybean extract; DM=diabetes mellitus
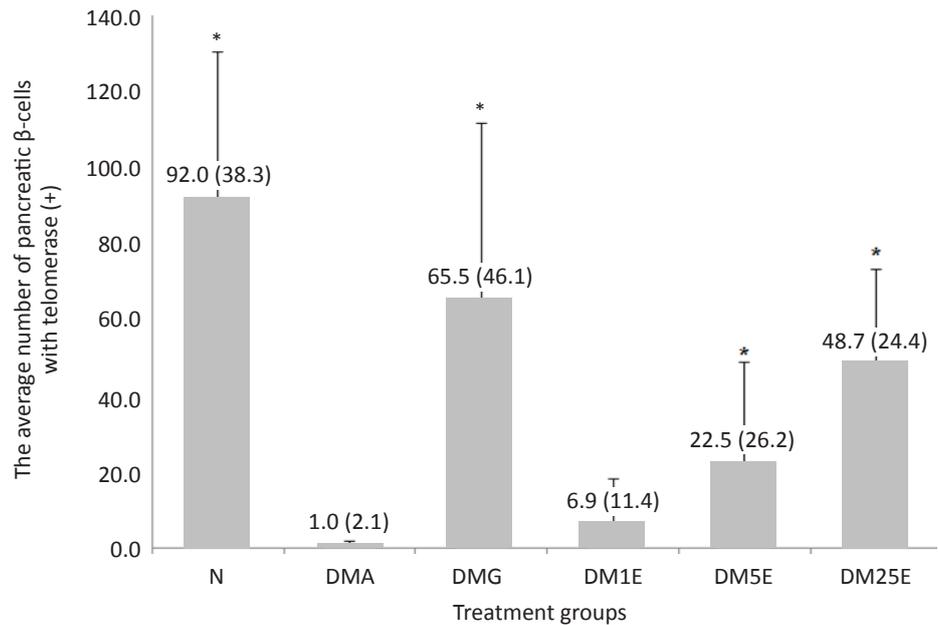
several mechanisms. That is, 1) it serves as an antioxidant that inhibits enzymes involved in ROS development (i.e., microsomal monooxygenase, glutathione S-transferase, nicotinamide adenine dinucleotide-hydrogen oxidase, etc.), or it acts as chelating element involved in the development of free radicals; 2) it serves as scavengers of ROS; and 3) it increases the regulation or provides protection for antioxidants.[16]

The TERT protein expression of the islets of Langerhans in rats supplemented with soybean extracts was much improved compared to rats without the soybean extracts. It has been well established that diabetes causes increased ROS production. Moreover, ROS causes TERT to flow out of the nucleus and into the cytoplasm. There is significantly increased ROS production in endothelial cells that results in reduced TERT activity in the nucleus. Meanwhile the TERT activity in the cytoplasm is increased. It indicates that the movement of TERT from the nucleus into the cytoplasm causes reduced its activity.[9]

DM rats supplemented with soybean extracts were more likely to have lower ROS (malondialdehyde) levels compared to DM rats without soybean extracts. This may have occurred since soybean extracts contains flavonoid, which serves as an antioxidant by inhibiting enzymes involved in ROS production; and as a scavenger or binder of metal ion that plays a role in the development of free radicals.[16,17] Reduced ROS level can prevent the outflow of TERT from the nucleus

into the cytoplasm. These findings are consistent with the results of Haendeler et al,[9] which found that the supplementation of the antioxidant, N-acetyl cysteine (NAC), in human endothelial cell cultures can block the outflow of TERT from the nucleus. Therefore, antioxidants may inhibit the outflow of TERT from the nucleus of pancreatic β-cells. The presence of TERT inside the cell is visualized by its expression in pancreatic β-cells following IHC staining.

TERT is a gene that codes for the catalytic subunit of telomerase, which serves to lengthen the end of telomeres using the TERC RNA subunit as a template. Increased telomerase activity or TERT mRNA expression has been detected in up to 90% in cancer cells. IHC staining for TERT in the nucleus that resulted in a brown color is considered as positive for telomerase, while nucleus with less color is considered as negative for telomerase.[18] The study demonstrated that there were a greater number of cells positive for telomerase in non-DM rats (negative control), the positive control, and groups treated with soybean extract compared to DM rats. In contrast, the highest number of cells negative for telomerase was found in DM rats compared to all other groups, and these differences were significant. The different number of TERT between DM rats and the positive control (i.e., glibenclamide) was caused by the glibenclamide that stimulates cells to secrete insulin.[19] Therefore, the glucose metabolism was better compared to DM rats. Normal blood glucose

levels results in reduced ROS levels. Thus, TERT does not flow from the cell nucleus. It may lead to a greater maintenance effect on the number of pancreatic β-cells positive for telomerase than DM rats.

Differences in the number of cells with or without telomerase may be caused by the antioxidant effects of genistein and daidzein. Antioxidant supplementation in DM rats can inhibit enzymes that play role in ROS production by sweeping ROS away and preventing the development of free radicals.[16] There is a balance between oxidants and antioxidants in the groups of rats treated with the soybean extract, which prevents the movement of TERT into the cytoplasm that leads to cell repair. This study is consistent with that of Kuhlow et al,[6] which suggests that telomerase deficiency (in rat genotypes negative for TERC) results in reduced cell mass because the regenerative capacity of pancreatic islets has been disturbed.

Increased ROS in DM patients is associated with telomere shortening. The level of telomere shortening strongly depends on oxidative induction and the balance of cellular oxidant. There are at least two mechanisms of ROS causing telomere shortening. First, telomere DNA is very susceptible to oxidative damage on GGG sequences. The sequence of 5' guanine GG and GGG are more susceptible to oxidation than single guanine in DNA, and 5' guanine GGG is more susceptible to oxidation than 5' guanine GG.[20] Second, increased oxidative stress can cause the nuclear TERT to come out into the cytoplasm through the nucleus pores. This was supported by the findings of Haendeler et al,[9] which demonstrated that hydrogen peroxide (H_2O_2) treatment on human kidney embryo cells caused reduced activity in nuclear TERT. In contrast, there is an increased of TERT activity in the cytoplasm. Ahmed et al[21] also confirmed that H_2O_2 concentration affects the expression of nuclear TERT and under chronic hyperoxia, the TERT gradually moves into the cytoplasm in the cell culture of human embryo lung fibroblasts. The results of this study showed that there was reduced nuclear TERT in the pancreatic β-cells of DM rats because it has flowed from the nucleus. To confirm the results, the cells positive and negative for TERT and TERT in DM rats were counted. The results of this counting showed a greater number of cells negative for TERT than positive. Subsequently, the DM rats were treated the soybean extract, which demonstrated that there was

greater number of cells positive for TERT compared to negative with significant differences (particularly at 25 ml/kgBW/day).

Hence, increased ROS in patients with DM may result in suboptimal functioning of telomerase.[22] Changes in the location of TERT in cell nuclei also occurred. It was demonstrated by the higher number of cells positive for TERT in DM rats treated with soybean extract compared to DM rats without treatment. According to Ahmed et al,[21] TERT will come out of the cell nucleus when the ROS level in the nucleus is increased, but this is reversible. That is, when the oxidative stress returns to normal it will reenter the cell nucleus. A study by Haendeler et al[9] showed that NAC supplementation on the cell culture of human endothelial cell can block the outflow of TERT and subsequently prevent the development of cellular aging.

This study demonstrated that soybean extract supplementation, particularly at a dose of 25 ml/kgBW/day, can inhibit the outflow of nuclear TERT of pancreatic β-cells and restore telomerase function to increase the number of pancreatic β-cells. Increasing the amount of TERT in pancreatic β-cells may have some effects on the function of the cells to secrete insulin. It is assumed that the antioxidant effect of the soybean extracts (that restores the number of β-cells) occurs through two mechanisms; 1) by reducing the ROS level allows the repair of damaged telomere DNA; 2) by inhibiting the outflow of nucleus TERT into the cytoplasm cellular aging can be delayed.[9,16]

The first limitation of this study was that rat models may not achieve the same results in human diabetes. Ideally, the study would be conducted on genetically modified diabetic rats. However, using this model organism would be unreasonably expensive. The second limitation is that the assessment of changes in the telomere should include measurements of telomere length but was not conducted here due to time constraints.

The soybean extracts was chosen in the present study because soy-based processed foods were widely used by Indonesians; and our previous experience have shown its beneficial effect in other diabetes models. Several other studies also supported this notion.

From the results of this study, it can be concluded that soybean extract supplementation can reduce FBG

levels and increase the expression of TERT protein in pancreatic β-cells. Moreover, this supplementation can also increase the number of pancreatic β-cells in diabetic rats.

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