J Med Sci
Volume 45, No.1, March: 1 - 11

Expression of receptor advanced glycosilation end product (RAGE) and active caspase-3 of the streptozotocin-induced chronic diabetes mellitus Sprague Dawley rats' sperm with soybean (*Glycin max*) powder suspension treatment

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ABSTRACT

Diabetes mellitus (DM) affects all the process of spermatogenesis. Chronic hyperglycemia in DM increases the expression of receptor for advanced glycosilation end products (RAGE) that is responsible for the activation of signal production of reactive oxygen species (ROS) and caspase 3. Active caspase 3 plays an important role in cell apoptosis. Soybean (*Glycin max*) is reported to have antihyperglycemic and antiadvanced glycosilation end products (antiAGE) and antioxidants activities. The aim of this study was to evaluate the effect of soybean powder suspension on the expression of RAGE and active caspase 3 of diabetic rats' sperm. This was an experimental study with post test only control group design using 30 male Sprague Dawley rats, aged 11-12 weeks old and weighed 200-250g. The rats were divided into five groups with six rats in each group. Group 1 was non diabetic rats and Group 2 was diabetic rats that were given aquadest. Group 3-5 were diabetic rats that were given a soybean powder suspension at dose of 400; 800 and 1600 mg/kg body weight (BW)/day, respectively. Diabetic rats were made by induction of a single intraperitoneal injection of streptozotocin (STZ) at a dose of 60 mg/kg BW. Soybean powder suspension was ingested for four weeks after 14 days STZ induction. Blood glucose levels were monitored before and three days after STZ induction and four weeks after suspension ingestion. The expression of RAGE and active caspase-3 were analyzed using immuno-histochemistry method four weeks after suspension ingestion. The results showed that soybean powder suspension ingestion significantly decreased blood glucose level of diabetic rats toward normality ($p<0.05$). However, the expression of RAGE and active caspase-3 in diabetic rats' sperm were not significantly lower than those after suspension ingestion. In conclusion, soybean powder suspension does not significantly affect the expression of RAGE and active caspase-3 in diabetic rats' sperm.

ABSTRAK

Diabetes melitus (DM) mempengaruhi semua proses spermatogenesis. Hiperglikemia kronis pada DM meningkatkan ekspresi *receptor for advanced glycosilation end products* (RAGE) yang bertanggung jawab terhadap aktivasi sinyal produksi *reactive oxygen species* (ROS) dan caspase-3. Caspase-3 aktif berperan penting pada apoptosis sel. Kedelai (*Glycin max*) dilaporkan memiliki aktivitas antihiperglikemia, *antiadvanced glycosilation end products* (antiAGE) dan antioksidan. Tujuan penelitian ini adalah mengevaluasi efek suspensi bubuk kedelai terhadap ekspresi RAGE

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dan caspase-3 aktif pada sperma tikus diabetes. Penelitian ini merupakan penelitian eksperimental dengan rancangan *post test only control group* menggunakan 30 ekor tikus jantan Sprague Dawley, umur 11-12 minggu dan berat 200-250 g. Tikus dibagi menjadi lima kelompok dengan masing-masing kelompok 6 ekor. Kelompok 1, tikus tidak DM dan Kelompok 2, tikus DM yang diberi akuades. Kelompok 3-5, tikus diabetes yang diberi suspensi bubuk kedelai dengan dosis berturut-turut 400; 800 dan 1600 mg/kg BB/hari. Tikus dibuat diabetes dengan diinduksi streptozotosin (STZ) dosis 60 mg/kg BB. Suspensi bubuk kedelai diberikan selama empat minggu hari ke 14 setelah induksi STZ. Kadar gula darah ditetapkan sebelum dan tiga hari setelah induksi STZ serta setelah empat minggu pemberian suspensi. Ekspresi RAGE dan caspase-3 aktif dianalisis menggunakan metode imunohistokimia setelah empat minggu pemberian suspensi. Hasil penelitian menunjukkan bahwa pemberian suspensi bubuk kedelai secara nyata menurunkan kadar gula darah hingga kadar normal ($p<0.05$). Namun demikian, ekspresi RAGE dan caspase-3 aktif sperma tikus diabetes lebih rendah tidak nyata dibandingkan setelah pemberian suspensi. Dapat disimpulkan, suspensi bubuk kedelai tidak mempengaruhi secara nyata ekspresi RAGE dan caspase-3 aktif pada sperma tikus diabetes.

Keywords: diabetes mellitus - sperm - soybean - RAGE - caspase-3

INTRODUCTION

The quality of sperm is one of the important biomarkers of the overall men's health. High-quality sperm indicates good health and optimally hormonal regulation. Poor-quality sperm is associated with an increased infertility,[1] testis malignancy,[2] and male mortality.[3] The main factor decreasing quality of sperm is an increase reactive oxygen species (ROS) which can be triggered by diabetes mellitus (DM).[1,4]

Diabetes mellitus causes impairment in all stages of spermatogenesis due to hyperglycemia. Hyperglycemia increases the expression of receptor for glycosilation advanced end products (RAGE) that is responsible for the activation of ROS generation and sperm apoptosis signal.[5] The increase of RAGE expression in DM compared to basal state is stimulated by the increase of advanced glycosilation end products (AGE) as a result of non-enzymatic glycosylation of glucose that occurs due to hyperglycemia.[6] The binding of AGE to RAGE increases the formation of ROS, nitrit oxide (NO), and ceramides as well as mitogen-activated protein kinases (MAPKs) such as JNK and p38 that can paticipate in apoptosis through induction of proapoptotic transcription factors forkhead box 1 (FOXO1) and caspase-3.[7]

Active caspase-3 is a major component of the apoptotic cascade that activates caspase-activated DNAase and induces DNA fragmentation lead to complete sperm death.[8,9] Inhibition of AGE formation in DM patients is expected to decrease RAGE and caspase-3 expression and reduce sperm apoptosis.

Soybean (*Glycine max*) has been reported to have antihyperglycemic and antiAGE activity. However, its mechanism has not been clearly described, yet.[10,11] Chang *et al*.[12] reported that administration of soybeans reduced fasting and post prandial glucose, improved catalase, and glutathione peroxidase (GPX) patients with type-2 DM. Furthermore, soybean was reported to be able to improve blood glucose levels, reduce ROS formation and DNA fragmentation of diabetic rats' sperm.[11] This study was conducted to investigate RAGE and active caspase-3 expressions in sperm of diabetic rats induced by streptozotocine after administration of soybean powder suspension.

MATERIALS AND METHODS

Soybean powder suspension preparation

Five hundred grams of soybeans obtained from a traditional market in Karangkajen, Yogyakarta were washed and dried using an

oven at temperature of 50 °C for 30 minutes. Dried soybeans were then powdered using a blender and then each dose of suspension administration i.e. 400; 800 and 1600 mg/kg body weight (BW) was made by adding aquadest until the volume of 2 mL.

Animals

This was an experimental study with post test only control group design. Thirty male Sprague Dawley rats, aged 11-12 weeks old, weighed 200-250g, obtained from LPPT (*Laboratorium Pengujian dan Penelitian Terpadu*/Integrated Research and Testing Laboratory), Universitas Gadjah Mada, Yogyakarta were used in this study. The rats were divided randomly into five groups with six rats in each group. Group 1 as normal or non diabetic rats control group and Group 2 as diabetic rats control group were given aquadest. Group 3-5 as treatment groups were given a soybean powder suspension dose of 400; 800 and 1600 mg/ kg body weight (BW)/day, respectively. Each rat was placed at individual cage and was housed at room temperature under 12 hour cycles of dark and light and was allowed standard food and water *ad libitum*. This study has received the approval from the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Induction of diabetes mellitus and treatment

Rats were induced with a single intraperitoneal injection of STZ [streptozotocin or 2-deoxy-2-(3-methyl-3-nitrosurea) 1-D-glucopyranose from MP Biomedical LCC, UK,] at a dose of 60 mg/kg BW in 0.5 mL of 100 mM acid citrate at pH 4.5. In the following 24 hours, and orally fed with glycose solution to avoid hypoglycemia resulting from massive β-cells destruction and intracellular insulin release due to STZ induction. Diabetic rats were confirmed if blood glucose level was >200 mg/dL .

Two weeks after diabetic rats were confirmed, the treatment groups (Group 3-5) were ingested soybean powder suspension using gastric cannula for four weeks according to each dose group. Normal rats control (Group 1) and diabetic rats control (Group 2) were ingested aquadest. Four weeks after soybean powder suspension ingestion, blood glucose level was measured followed by termination of the rats.

Venous blood samples were taken from the orbital sinus using heparinized capillary glass and collected in EDTA anticoagulant tubes. The tubes were kept at room temperature for 15 minutes and centrifuged at 250 g for 10 minutes at room temperature to obtain serum samples. Blood glucose level of the serum samples was then measured by the enzymatic GOD-PAP method using Diasys GOD-PAP kit (Diasys Diagnostic Systems GmbH, Germany).

Animals were sacrificed four weeks after blood glucose level measurement using CO_2 after an intraperitoneal injection of pentothal at dose of 40 mg/kg BW. A transvere abdominal incision was made and testis with epididymis and its vas deferen were removed from the rats. The epididymis was separated from the testis and cut using scissors into small pieces. The epididymal pieces were put in 2 mL warmed rats' sperm isolation medium containing 17 mM $NaHCO_3$, 96.4 mM NaCl, 4.76 mM KCl, 21.58 mM Na-lactate, 5.56 mM glucose, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 0.5 mM Na-pyruvate, 4.0 mg/mL BSA, 50 mg/mL streptomycin, 75 mg/mL penicillin, 20 mM HEPES, 5 mg/mL phenol red at pH 7.3. The epididymal pieces were then centrifugated at 300 g for 10 minutes and the seminal plasma was removed. Pellets were then washed in PBS

(phosphate-buffered saline from Sigma Aldrich, Poole, UK) with the same volume and recentrifugated. After discarding the supernatant, the pellets were resuspended in PBS and 10 µL of the pellets was removed to prepare sperm smears for immunohysto-chemistry examination. Sperm smears were prepared on 3-aminopropyltriethoxysilane (APE) coated microscope slides, left to air dry, then fixed in absolute ethanol, air dried and stored until use.

Immunohistochemical analysis of RAGE expression

The RAGE expression was evaluated using immunohistochemistry method as conducted by Mallidis *et al.*[5] with modification. Briefly, antigen unmasking was performed by incubating the sperm smears slides with a 50% trypsin : 50% vercene solution for 2 minutes. After being washed in water for 20 minutes, the slides were then blocked with endogenous peroxidase by incubating with a 3% H_2O_2 : 0.02% avidin for 5 minutes and then rinsed in PBS 3 times. The slides were then incubated with primary monoclonal antibody anti-mouse RAGE (catalog number MAB1179 R & D system) (25 µg/mL) overnight at 4°C. Following the incubation, the slides were washed in PBS and then incubated with a biotinylated secondary antibody for 30 minutes and washed again in PBS. The slides were incubated with a streptavidine peoxidase for 30 minutes at room temperature, washed in PBS and incubated with DAB (diamino-benzidine) for 5-10 minutes. The slides were then lightly countersatined by haematoxylin, incubated for 30 second at room temperature and washed in water. The slides were then dried and coverslipped. All slides were then examined and evaluated using light microscope. The RAGE expression in spermatozoa was identified by a brown color of the cell, while a blue color of a cell indicated no expression of the RAGE. The RAGE expression was observed on 100 spermatozoa and its percentage was calculated.

Immunohistochemical analysis of caspase-3 expression

The active caspase-3 expression was evaluated using immunohistochemistry method as conducted by Cayli *et al.*[13] with modification. Briefly, antigen unmasking was performed by incubating the sperm smears slides with a 50% trypsin : 50% vercene solution for 2 minutes. After being washed in water for 20 minutes, the slides were then blocked with endogenous peroxidase by incubating with a 3% H_2O_2 : 0.02% avidin for 5 minutes and then rinsed in PBS 3 times. The slides were then incubated with primary polyclonal antibody Anti-ACTIVE® caspase-3 pAb (catalog number G7481 Promega) (25 µg/mL) overnight at 4°C. The next day after incubation, the slides were washed in PBS and then incubated with a biotinylated secondary antibody for 30 minutes at room temperature. After rewashed in PBS, the slides were incubated with a streptavidine peoxidase for 30 minutes, rinsed in PBS and incubated with DAB for 10 minutes. The slides were then lightly countersatined by haemato-xylin, incubated for 30 seconds at room temperature and washed in water three times. The slides were then dried and coverslipped. All slides were then examined and evaluated using light microscope. The active caspase-3 expression in spermatozoa was identified by a brown color of the cell, while a blue color of a cell indicated no expression. The active caspase-3 expression was observed on 100 spermatozoa and its percentage was calculated.

Statistical analysis

Data were presented as mean ± standard deviation (SD) and analyzed using SPSS program. The difference of blood glucose

levels, RAGE and active caspase-3 expressions on spermatozoa between treatment groups with control groups were analyzed using Kruskal-Wallis test. The significant differences between groups were analyzed with Mann-Whitney test. A p value of less than 0.05 was accepted as statistically significant.

RESULTS

Blood glucose level before, three days after STZ induction and after four weeks treatment with soybean powder suspension and number of surviving rats of each group were presented in TABLE 1. The blood glucose levels three days after STZ induction reached > 200 mg/dL indicating that the induction successfully created diabetic rats. Moreover, ingestion of soy powder suspension during four weeks significantly decreased blood glucose levels ($p<0.05$) with the highest decrease achieved at a dose of 1600 mg/kg BW/day (179.3 ± 2.3 mg/dL).

TABLE 1. Blood glucose level and number of surviving rats in non diabetic rats and diabetic rats after four weeks of soybean powder suspension ingestion

Groups	n	Blood glucose level (mg/dL) Mean ± SD		
		Before STZ induction	3 days after STZ induction	4 weeks after treatment
1. Non DM + aquadest	4	74.8 ±1.0	75.7 ±1.3	78.4 ±1.3
2. DM + aquadest	3	76.2 ±1.8	219.8 ±2.4	212.4 ±15.7
3. DM +dose 400mg/kgBW/day	5	76.4 ±1.4	213.4 ±6.8	188.6 ±1.8 *
4. DM +dose 800mg/kgBW/day	5	75.2 ±1.7	219.6 ±5.9	184.7 ±1.5 *
5. DM +dose 1600mg/kgBW/day	5	74.8 ±0.9	220.4 ±4.5	179.3 ±2.3 *

* Uji *Mann-Whitney test, significantly different ($p<0.05$)*

The immunohistochemistry staining of the sperm smears slides with primary anti-RAGE antibody is shown in FIGURE 1. The positive RAGE expression was characterized by brown pigments of the spermatozoa (FIGURE 1A). The RAGE distributed in the acrosome and midpiece of spermatozoa. The negative RAGE expression (FIGURE 1B) as well as negative control without primary antibody (FIGURE 1C) were characterized by blue pigments of the spermatozoa.

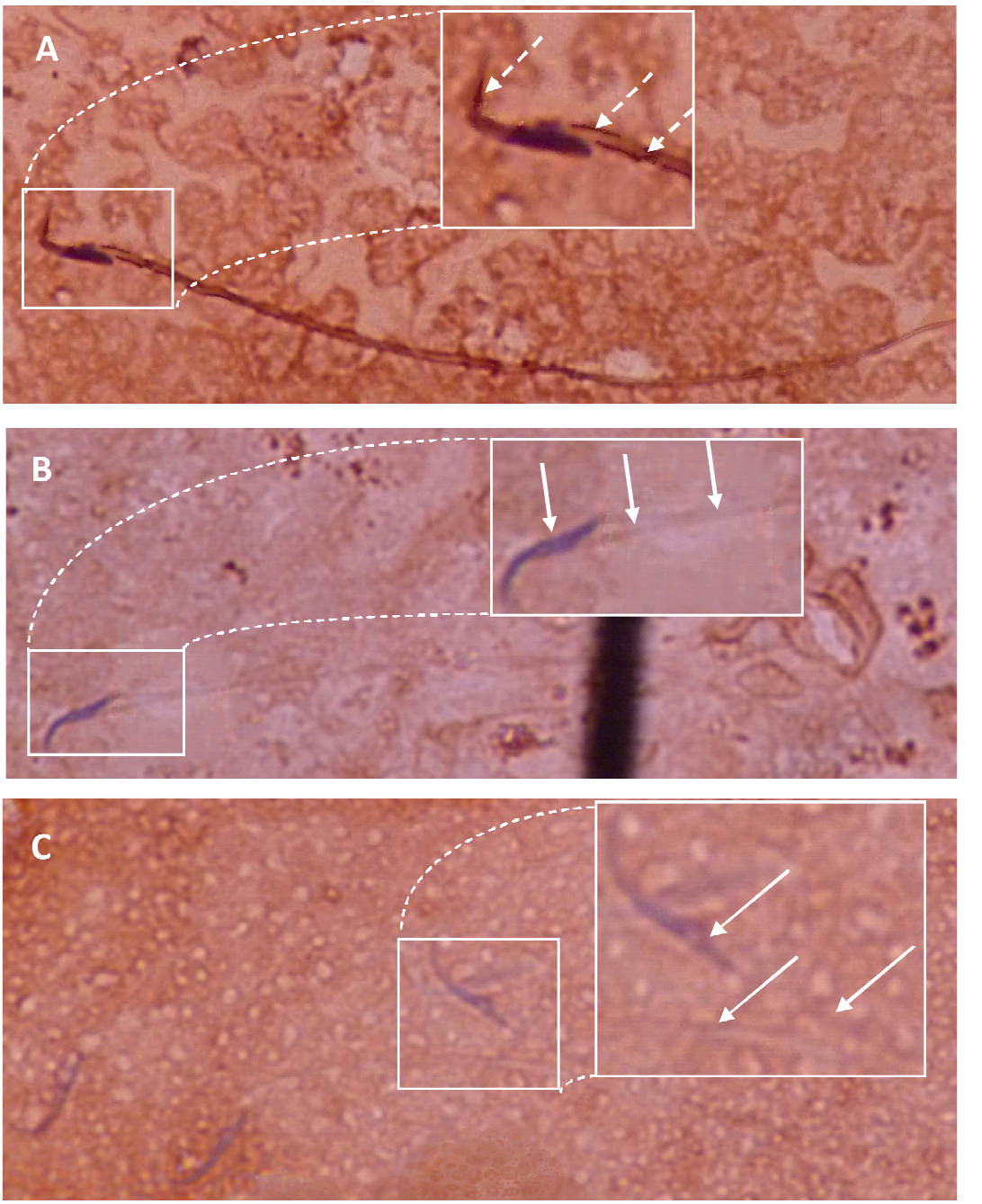

FIGURE 1. Expression of RAGE on diabetic rats' sperm after four weeks of soybean powder suspension ingestion. A. Positive RAGE expression (arrow heads) is shown by brown pigments. B. Negative RAGE expression (arrow head) and C. Negative control (arrow head) is shown by blue pigments. Magnification of 800x.

The percentage of RAGE expression in rats' sperm of each group is presented in TABLE 2. The expression of RAGE in diabetic rats' sperm (64.5 ± 5.4%) was higher than normal rats (23.6 ± 3.3%) ($p<0.05$). The expression of RAGE in diabetic rats' sperm in all three dose groups soybean powder suspension ingestion (400; 800 and 1600 mg/kg BW/day) was lower than the DM group. The lowest expression of RAGE was observed in dose of 1600 mg/kg BW/day (54.6 ± 7.3%). However, it was not statistically and significantly different ($p> 0.05$).

TABLE 2. The mean and median values of RAGE expression in non diabetic rats sperm and diabetic rats sperm after four weeks of soybean powder suspension ingestion

Groups	n	Expression of RAGE (%)	
		Mean ± SD	Median
1. Non DM + aquadest	4	23.6 ±3.3*	23.4
2. DM + aquadest	3	64.5 ±5.4	65.0
3. DM +dose 400mg/kgBW/day	5	59.4 ±5.5	59.7
4. DM +dose 800mg/kgBW/day	5	56.9 ±6.7	56.1
5. DM +dose 1600mg/kgBW/day	5	54.6 ±7.3	55.3

* *Mann-Whitney test, significantly different ($p<0.05$)*

The immunohistochemistry staining of the sperm smears slides with primary anti-active caspase-3 antibody is shown in FIGURE 2. The positive active caspase-3 expression was characterized by brown pigment (FIGURE 2A). The active caspase-3 was distributed in the head and midpiece of diabetic rats' sperms. The negative active caspase-3 epression (FIGURE 2B) as well as negative control without primary antibody (FIGURE 2C) were characterized by blue pigments.

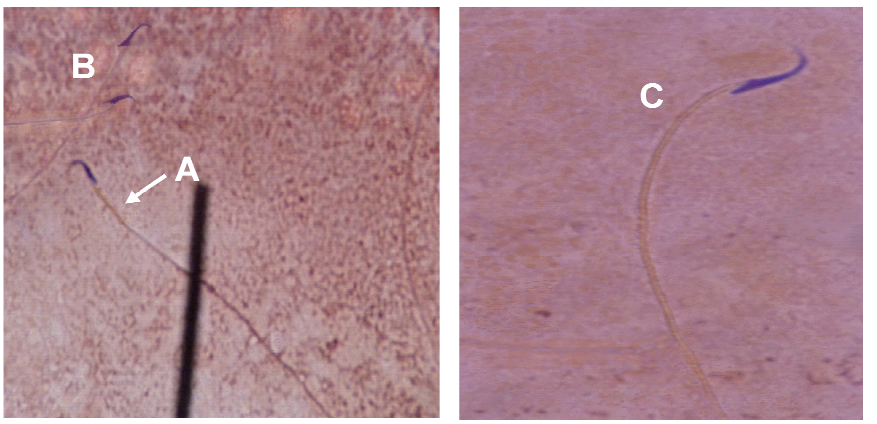

FIGURE 2. Expression of active caspase-3 on diabetic rats' sperm after four weeks of soybean powder suspension ingestion. A. Positive active caspase-3 expression (arrow heads) is shown by brown pigments. B. Negative active caspase-3 expression and C. Negative control is shown by blue pigments. Magnification of 800x.

The percentage of active caspase-3 expression in rats' sperm of each group is presented in TABLE 3. The expression of active caspase-3 in diabetic rats' sperm (16.2 ± 5.6%) was higher than normal rats (6.8 ± 1.0%) ($p<0.05$). The expression of active caspase-3 in diabetic rats' sperm in all three dose groups soybean powder suspension ingestion (400; 800 and 1600 mg/kg BW/day) were lower than the DM group. The lowest expression of active caspase-3 was observed in dose of 1600 mg/kg BW/day (14.5 ± 2.6%). However, it was not statistically and significantly different ($p>0.05$).

TABLE 3. The mean and median values of active caspase-3 expression in non diabetic rats sperm and diabetic rats sperm after four weeks of soybean powder suspension ingestion

Groups	n	Expression of active caspase-3 (%)	
		Mean ± SD	Median
1. Non DM + aquadest	4	6.8 ±1.0 *	6.7
2. DM + aquadest	3	16.2 ±5.6	15.5
3. DM +dose 400mg/kgBW/day	5	15.4 ±2.3	15.3
4. DM +dose 800mg/kgBW/day	5	14.6 ±2.2	14.8
5. DM +dose 1600mg/kgBW/day	5	14.5 ±2.6	14.2

* *Mann-Whitney test, significantly different ($p<0.05$)*

DISCUSSION

Diabetes mellitus is a multi-systemic disease characterized by hyperglycemia with numerous complications. Hyperglicemia leads to structural and functional changes in various target tissues and organs including reproductive system.[14,15] Experimentally, the structural and functional changes of the tissues and organs in diabetic male rats are observed 14 days after diabetes occured.[14] Therefore, in this study, soybean powder suspension was given 14 days after STZ induction which suspected that histological changes of testicular tissue of diabetic rats could be observed. The results showed that the blood glucose level, the expressions of RAGE as well as active caspase-3 of diabetic rats sperm were significantly higher than those in non diabetic rats (TABLE 1-3). It was indicated that the histological changes of testicular tissue had been occured.

Soybean powder suspension ingestion at dose 400; 800 and 1600 mg/kg BW/day for four weeks on diabetic rats could significantly decrease the blood glucose level towards normality (<200 g/dL) ($p<0.05$; TABLE 1). The highest effect was achieved at dose ingestion of 1600 mg/kg BW/day. The effect of soybean on glycemic control both in animal model and human has been proven by some authors. Soybean powder suspension ingestion decrease blood glucose level and DNA fragmentation on male diabetic rats.[11] Moreover, soybean consumption improves glycemic control and prevents diabetic nephropathy progression in rats.[16] It is also reported that soybean fibers can control blood glucose level, blood-lipid level by improving their metabolisms which then leads to protect liver and renal damage of diabetic mice.[17]

The RAGE expression as well as active caspase-3 in diabetic rats sperms group (Group 2) in this study was significantly higher than in non diabetic or normal rats sperms group (Group 1) ($p<0.05$; TABLE 2 and 3). This result was similar with previous reports. The RAGE is normally expressed in normal cells to response endogenous ligands. However, in pathological conditions such as DM, the RAGE expression increases to maintain homeostasis mechanism in response to excessive tissue damage including sperm damage.[6] Hyperglycemia in DM increases the RAGE expression which leads to the activation of ROS generation and causes sperm oxidative damage.[5] Furthermore, the increase of RAGE expression stimulates ROS generation and apoptosis signaling pathway such as MAPKs, RAC-1, PI3kinase, JAK/STAT, and NFkB.[7] ROS causes leakage citokrom C from mitochondria activating the mitochondrial pathway through the activation of caspase 9. Moreover, ROS activates the cytoplasmic pathway through the activation of caspase 8. ROS together with ceramid and NO activate JNK and p38 which in turn induce FOXO1 and caspase-3.[7] Activation of caspase-3 is an important role in the apoptotic cascade. After caspase-3 becomes active, apoptotic signals can not be stopped, therefore the apoptosis continues to end.[9]

Soybean contains rich isoflavones, arginine and selenium which have some biological activities including antihyperglicemic, antiAGE, and estrogenic. Glyceollins, soy isoflavone phytoalexins, improve oral disposal in prediabetic rats.[18] Biochanin A that is isolated from soybean shows antihyperglicemic effect on STZ-diabetic rats.[19] Genestein, another soy isoflavone, has AGE inhibitor activity by capturing methyl glioksal that leads to the inhibition of the formation AGE.[20] Moreover, genestein regulates antioxidant genes expression involving estrogen receptors, ERK1/2 and NFκB.[21]

This study showed that the RAGE expression as well as active caspase-3 in diabetic rats' sperm after soybean powder suspension ingestion at dose 400, 800 and 1600 mg/kg BW/day for four weeks (Group 3-5) was lower than in diabatic rats sperms without those suspension ingestion (Group 2) ($p>0.05$; TABLE 2 and 3). However, it was not significantly different indicating that soybean did not significantly affect the expression of RAGE and active caspase-3 in diabetic rats sperms.

Some factors may contribute in the effect of soybean on the expression of RAGE and active caspase-3 in diabetic rats sperms such as level of blood glucose level, level of follicle stimulating hormone (FSH), testoterone and estrogen intratestticular. Although the blood glucose level significantly decreased towards normality after soybean powder suspension ingestion, it was still much higher (> 179 g/dL) compared to the blood glucose level of normal rats (<78.4 g/dL) (TABLE 1). This high blood glucose level may still generate ROS and increase the RAGE expression. In this study, serum FSH, testoterone and estrogen level were not monitored. However, it is reported that the low in FSH, testoterone and estrogen level can cause sperm immaturity.[22-24] Immature spermatozoa is a source of ROS and lack of antiapoptotic factor Bcl-xl.[13]

CONCLUSION

In conclusion, soybean powder suspension ingestion decreases blood glucose level of diabetic rats normally. However, it does not have significant effect on the expression of RAGE as well active caspase 3 in diabetic rats' sperm.

ACKNOWLEDGEMENTS

The authors would like to thank Dra. *Dewajani Purnomosari*, M.Si., Ph.D, Mrs. Yati and Mrs. Wiwid from Histology Laboratory, Faculty of Medicine, Universitas Gadjah Mada, and Mrs. Agustin from Pathology Anatomy Laboratory, Dr. Sardjito General Hospital for their technical assistance in immunohistochemistry staining. We would also like to thank Mr. Parno and Mr. Wakidi from Physiology Laboratory, Faculty of Medicine, Universitas Gadjah Mada for their assistances in animal care.

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