J Med Sci
Volume 44, No.1, March 2012: 18 - 25

Serum testosterone level and active caspase-3 of Leydig cells of diabetic Sprague-Dawley male rats after administration of soybean (*Glycin max*) powder suspension

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ABSTRACT

Diabetes mellitus (DM) is known as the main death cause in the world. This disease causes acute and chronic complication. The common chronic complication on male reproduction system is the decrease of testosterone level. This hormone is produced in the Leydig cells. Soybeans (*Glycin max*) has been used in the management of DM to maintain blood glucose level. However, the effect of soybean on serum testosterone level is still unclear. The aim of this study was to evaluate serum testosterone level and caspase-3 active of Leydig cells of diabetic Sprague-Dawley (SD) male rats model induced by streptozotocine (STZ) after oral ingestion of soybean powder suspension. This was an experimental study with pre and post test control group design. Thirty SD male rats, aged 11-12 weeks with body weight (BW) of 200-250 g were divided into 5 groups with 6 rats in each group. The first group (G1) was normal rat control and the second group (G2) was diabetic rat control. The third to fifth group (G3, G4 and G5) were diabetic rat treatment with oral ingestion of soybean powder suspension that was given once a day during 4 weeks with dose of 400; 800 and 1600 mg/kg BW, respectively. The serum testosterone level was measured by an ELISA and active caspase-3 of Leydig cells were measured by an immunohistochemistry method. The result showed that the serum testosterone level and active casapse-3 Leydig cells of diabetic rats model induced by STZ were not significantly different after oral ingestion of soybean powder suspension with dose of 400, 800 and 1600 mg/kg BW ($p > 0.05$). In conclusion, soybean powder suspension did not affect the serum testosterone level and active caspase-3 Leydig cells in diabetic rats.

ABSTRAK

Diabetes melitus (DM) diketahui sebagai penyebab kematian utama di dunia. Penyakit ini dapat menyebabkan komplikasi akut maupun kronis. Komplikasi kronis yang umum terjadi pada sistem reproduksi laki-laki adalah penurunan kadar testosteron serum yang diproduksi oleh sel Leydig. Kedelai telah digunakan dalam pengelolaan DM untuk mengontrol kadar gula darah. Namun demikian bagaimana efek kedelai pada kadar testosteron serum belum jelas. Penelitian ini bertujuan untuk mengkaji kadar testosteron serum dan caspase-3 aktif di sel Leydig pada tikus jantan Sprague-Dawley (SD) diabetes yang diinduksi oleh streptozotocin (STZ) setelah pemberian oral suspensi serbuk kedelai. Penelitian ini merupakan penelitian eksperimental dengan rancangan *pre and post test control group.* Tiga puluh ekor tikus SD jantan berumur 11-12 minggu dengan berat badan (BB) 200-250 g dibagi menjadi 5 kelompok dengan 6 ekor tikus masing-masing kelompok. Kelompok pertama (G1) merupakan kontrol tikus normal, kelompok ke 2 (G2) kelompok

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kontrol tikus diabetes. Kelompok 3-4 (G3, G4 dan G5) adalah kelompok tikus diabetes perlakuan yang diberi suspensi serbuk kedelai sekali sehari selama 4 minggu dengan dosis berturut-turut 400, 800 dan 1600 mg/kg BB. Kadar testosteron serum ditetapkan dengan ELISA dan caspase-3 aktif sel Leydig ditetapkan dengan metode imunohistokemistri. Hasil penelitian menunjukkan kadar testosteron serum dan caspase-3 aktif sel Leydig tikus jantan SD diabetes yang diinduksi STZ tidak berbeda bermakna setelah pemberian oral suspensi serbuk kedelai dengan dosis 400, 800 dan 1600 mg/kg BB ($p > 0.05$). Dapat disimpulkan, suspensi serbuk kedelai tidak berefek terhadap kadar testosteron serum dan caspase-3 aktif sel Leydig tikus diabetes.

Keywords: diabetes - soybean - phytoestrogen - testosterone - active caspase-3

INTRODUCTION

Diabetes mellitus (DM), well known as *kencing manis* in Indonesia, has now become one of main death causes in the world.[1] Chronic diabetes can cause many complications including reproductive toxicity. Diabetes mellitus causes low serum testosterone level which is associated with sexual dysfunction and infertility.[2-4] In addition, the low serum testosterone level has also been associated with insulin resistance, hyperglycemic, and the increasing risk of blood vessel disease.[5,6] The low serum testosterone level in DM patients is caused by high activities of aromatase on body fat tissue which has an important role in the testosterone conversion to estradiol. Moreover, the increase of serum estradiol level can suppress hypothalamus hypophysis axis function in control of testosterone level.[3]

Hyperglycemic on DM can increase intracellular ROS (reactive oxygen species) in the body which plays an important role on diabetes complications including reproductive impairment.[7,8] The ROS is produced continuously by mitochondria of cells, electron transport reaction chain and metabolic process of Leydig cell. In addition, steroidogenesis cytochrom $P450_{arom}$ enzyme on the Leydig cell also produces ROS as catalytic reaction mechanism.[9] The increase of ROS excessively can inhibit cholesterol transport and steroidogenesis, as well as impair Leydig cells that makes the serum testosterone level decrease.[10,11] Moreover, the increase of ROS level can cause Leydig cell apoptosis in DM patients.[12]

Chronic diabetes needs an expensive and long treatment. According to WHO, the cost of complicated DM is US$ 46.207 per year.[13] Therefore, a comprehensive management of DM is needed to prevent the complication. Diet of food has been proposed in the management of DM. Soybean diet has been used by DM patients to maintain blood glucose level. Soybean is believed to be able to decrease blood glucose level, which is helpful in controlling this disease.[14,15] Soybeans also produce anti-oxidant enzymes which can minimize oxidative stress on type 2 DM patients.[16,17] Moreover, soybean contains high level of arginin and glycin, amino acids composed of insulin and glucagon.[14]

Although soybean is reported to be useful in maintaining blood glucose level, it is also reported to cause reproductive toxicity, especially due to the decrease in testosterone level. However, several studies concerning the toxicity of soybean on reproductive system provide different results. Soybean contains isoflavones phytoestrogen namely genistein and daidzein. The phytoestrogen is reported to be able to decrease serum testosterone level.[18,19] In contrast, Sherrill *et al.*[20] reported that isoflavones induced proliferative activity in Leydig cells which was induced in androgen biosynthesis and/or increased serum and testicular testosterone levels. This study was

conducted to evaluate the effect of soybean powder suspension on serum testosterone level of diabetic Sprague-Dawley male rats induced by streptozotocin. Moreover, the effect of soybean on expression of active caspase-3 of Leydig cells of the diabetic rats was also measured to evaluate the cells apoptosis.

MATERIALS AND METHODS

Animal and material

This was an experimental study with pre and post test control group design. Sprague-Dawley (SD) male rats, aged 11-12 weeks with body weight (BW) of 200-250 g obtained from the Integrated Research and Testing Laboratory, Gadjah Mada University were used in this study. Yellow soybean (*G. max*) was collected from a traditional market in Yogyakarta, while aquades and streptozotocin (STZ) were obtained from MP Biomedic Inc. The study had been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada.

Induction of diabetes in rats and treatment

Sixty male rats were divided into 5 groups with 6 rats in each group. The first group (G1) was nondiabetic rats as normal control and the second group (G2) was untreated diabetic rats as diabetes control. The third to fifth group (G3, G4 and G5) were treated diabetic rats with oral ingestion of soybean powder suspension that was given once a day during 4 weeks with dose of 400; 800 and 1600 mg/kg BW, respectively. Diabetes was induced by a single intramuscular injection of STZ at the dose of 60 mg/kg BW after 24 hours of fasting. Rats with fasting blood glucose of more than 200 mg/dL were considered as diabetic rats. Streptozotocin induces diabetes within 3 days by destroying the beta cells. The diabetic and nondiabetic rats were kept in cages individually and separately. During the study period, food and water intake were monitored.

Serum testosterone level assay

The serum testosterone level was measured by ELISA method before and after 2 weeks of STZ induction. Samples obtained by centrifugation of blood samples from sinus orbita were immediately analyzed or stored at -80 ^{o}C until analysis. Twenty µL of each standard, serum samples and controls were dispensed with new disposable tips into appropriate microtiter wells. One hundred µL of testosterone-HRP reagent was added into each well, as well as fifty µL of rat antitestosterone reagent. The solution in wells was mixed for 30 seconds to have completed mixing and was then incubated at room temperature for 60 minutes. The wells were washed 3 times with wash solution. One hundred µL of a solution of 3,3',5,5'-tetramethylbenzidine (TMB) was added into each well, gently mixed for 5 seconds and incubated at room temperature (18-26^{o}C) for 15 minutes. The reaction was stopped with the addition 50 µL of stop solution to each well. The solution was carefully mixed for 30 seconds until the blue color changed to yellow color completely. The color optical density was read with ELISA reader at 450 nm within 15 minutes. The serum testosterone level was directly proportional to the color optical density of the samples.

Animal termination

Termination process was executed by ether inhalation. Decapitation was performed after termination. After termination, left testis was collected gently by transversal incision on posterior abdomen and was fixed with PBS formalin 10%. Next step was the preparation of embedding paraffin and section was cut with 3 µm thickness.

Active caspase-3 assay

The active caspase-3 expression to describe apoptotic Leydig cell was measured by immunohistochemistry as described by Kim *et al.*[21] after oral ingestion of soybean powder suspension during 4 weeks. After deparaffinizing with xylol every 5 minutes and rehydration with ethanol, testis section was successively immersed in absolute ethanol twice every 3 minutes, ethanol 95% twice every 3 minutes and ethanol 70% for 3 minutes, and aquabidest. The section was then washed in PBS (phosphate buffer saline), added drops proteinase K and being left for 20 minutes. The section was incubated in PBS containing H_20_2 3% for 5 minutes to inhibit endogenous peroxide activities and washed in PBS for 15 minutes.

Blocking with normal goat serum 1.5% in PBS was conducted and followed by incubation with caspase-3 active anti-human polyclonal rabbit (0.5µg/mL) in normal goat serum 1.5% in PBS for 45% at room temperature. The section was then incubated on biotin-conjugated goat antirabbit IgG, avidin-biotyn-peroxide complex for 1 hour and diaminobenzinidine (DAB). Then it was counterstained with hematoxylin. On the negative control, primary antibody rabbit IgG 1 µg/mL was added. Observation and quantification of active caspase-3 Leydig cell were performed to transversal testis tissue. The positive active caspase-3 Leydig cell was characterized with brown color of nucleus, whereas the negative caspase-3 or normal Leydig cell was characterized with bluish color. Quantification of the positive active caspase-3 Leydig cell was conducted by randomizing of interstitial space (20 testis sections per slide) among 3 or 4 circular tubules. Images were captured using a Nikon Optiphot-2 microscope at 400 x magnification.

Statistical analysis

Statistical analysis was conducted using SPSS program. The means and standard deviations (SD) of serum testosterone level and active caspase-3 Leydig cell were calculated and the differences between five groups were analyzed with Kruskall-Wallis test. If the result showed a statistical significance, analysis would be followed by Wilcoxon signed rank test for serum testosterone level and by Mann-Whitney test for active caspase-3 Leydig cell.

RESULTS

Serum testosterone level of nondiabetic, untreated and treated diabetic rats during 4 weeks of study period is shown in TABLE 1. The serum testosterone level of untreated (G2) and treated (G3, G4 and G5) diabetic rats before and after 4 weeks study period were significantly lower than nondiabetic (G1) rats ($p<0.05$), whereas the serum testosterone level of treated diabetic rats with oral ingestion of soybean powder suspension that was given once a day during 4 weeks with dose of 400; 800 and 1600 mg/kg BW, respectively (G3, G4 and G5) were similar with untreated diabetic rats before and after 4 weeks of study period (G2) ($p>0.05$). After ingestion of soybean powder suspension, the serum level testosterone on treated diabetic rats (G3, G4 and G5) were higher than that before ingestion although it was not statistically significant ($p>0.05$). However, after 4 weeks of study period (after ingestion) the serum testosterone level on nondiabetic and untreated diabetic rats were lower than that before study period although it was not statistically significant either ($p>0.05$).

TABLE 1. Serum testosterone level of nondiabetic, untreated and treated diabetic rats before and after 4 weeks of study period

Group	n	Serum testosterone level (ng/mL)		p
		Before ingestion	After ingestion	
Nondiabetic rats (G1)	4	3.33±1.379	2.99±1.418	0.068
Untreated diabetic rats (G2)	3	0.28±1.379[a]	0.24±0.020[b]	0.285
Diabetic rats + dose 400 mg/kgBW (G3)	5	0.26±0.262[a]	0.29±0.044[b]	0.080
Diabetic rats + dose 800 mg/kgBW (G4)	5	0.27±0.043[a]	0.31±0.025[b]	0.138
Diabetic rats + dose 1600 mg/kgBW (G5)	6	0.29±0.12[a]	0.33±0.059[b]	0.463

a and b: significantly different with G1; n: number of sample

Active caspase-3 expression of Leydig cell between nondiabetic, untreated and treated diabetic rats before and after 4 weeks of study period is shown in TABLE 2. The active caspase-3 expression of Leydig cell of untreated diabetic rats (G2) and treated diabetic rats with oral ingestion of soybean powder suspension that was given once a day during 4 weeks with dose of 400; 800 and 1600 mg/kg BW, respectively (G3, G4 and G5) were significantly higher than nondiabetic rats ($p<0.05$). Meanwhile, the active caspase-3 expression of Leydig cell of treated diabetic rats (G3, G4 and G5) was lower than untreated diabetic rats although it was not significantly different ($p>0.05$). Moreover, the active caspase-3 of Leydig cell decreased proportionally as the soybean powder suspension dose increased although it was not significantly different either ($p>0.05$).

TABLE 2. Active caspase-3 expression of Leydig cell nondiabetic, untreated and treated diabetic rats before and after 4 weeks of study period

Group	n	Active caspase-3 (mean ⊥ SD)
Nondiabetic rats (G1)	4	0.6 ± 0.23
Untreated diabetic rats (G2)	3	2.6 ± 0.95[a]
Diabetic rats + dose 400 mg/kgBW (G3)	5	2.1 ± 0.72[a]
Diabetic rats + dose 800 mg/kgBW (G4)	5	2.0 ± 0.89[a]
Diabetic rats + dose 1600 mg/kgBW (G5)	6	1.8 ± 0.93

a: significantly different with G1; n: number of sample

DISCUSSION

This study showed that the serum testosterone level of diabetic rats (G2 to G5) was significantly lower than nondiabetic or normal rats (G1) indicating that diabetes causes serum testosterone level to decrease (TABLE 1). The decrease of serum testosterone level in diabetes has been well known both in diabetic animals model or human diabetes patients. Saad and Gooren[22] reported that men with diabetes have lower serum testosterone levels compared to men without a history of diabetes. Moreover, young type 2 DM patients have significantly lower plasma total and free testosterone level

when compared to type 1 DM patients of a comparable age.[3] Low blood insulin level causes carbohydrate metabolism disruption resulting in hyperglycemic. Chronic hyperglycemic causes free radical increase through various pathways resulting in oxidative stress which causes steroidogenic enzyme 17,20-lyase activities and dehydroepiandrossterone (DHEA) as testosterone hormone precursor to decrease. The decrease of DHEA causes the decrease of testosterone level.[23]

This study also found that the serum testosterone level of treated diabetic rats after oral ingestion of soybean powder suspension that was given once a day during 4 weeks in each dose were higher than before ingestion although it was not statistically different (TABLE 1). This study suggested that soybean might not cause reproductive toxicity as it was indicated that there was no change in serum testosterone level after soybean powder suspension ingestion.

The effect of soybean ingestion in the serum testosterone level of diabetes patients is still unclear. Soybean contains isoflavone phytoestrogen namely genistein and daidzein. Phytoestrogen has been reported to be able to decrease serum testosterone level by converting cholesterol delivery into steroidogenesis pathway and influence androgen biosynthesis by inhibitting 5á-reductase-enzyme and 17â-hydrosteroid dehydrogenase.[18,19] Inversely, Sherrill *et al.*[20] reported that isoflavones induced proliferative activity in Leydig cells by regulating Leydig cell division which involved express estrogen receptors (ESRs), acting in concert with signaling molecules in the transduction pathway mediated by protein kinase B (AKT) and mitogen-activated protein kinase (MAPK). Enhanced proliferative activity increased Leydig cell numbers, which induced in androgen biosynthesis and/or increased serum and testicular testosterone levels.

The expression of active caspase-3 of Leydig cells increased significantly in untreated and treated diabetic rats (G2 to G5) when compared to normal or nondiabetic rats ($p<0.05$) indicating that the diabetes caused Leydig cells apoptosis (TABLE 2). Caspase-3 is a caspase protein that plays an important role in the execution-phase of cell apoptosis. The apoptosis of Leydig cells caused by diabetes has been reported by some authors. Ahmed[7] reported that hyperglycemic on DM can increase intracellular ROS which plays an important role on diabetes complications.[8] In relation to reproductive tissue complication, the increase of ROS level on DM can cause Leydig cells apoptosis.[12]

The expressions of active caspase-3 of Leydig cells of treated diabetic rats (G3, G4 and G5) were lower than untreated diabetic rats although it was not significantly different (TABLE 2). It was indicated that soybean ingestion on diabetic rats could prevent the further apoptosis of Leydig cells or infertility caused by diabetes. The different result has been reported by Chavarro *et al.*[24] who showed that isoflavones has been related to male reproductive disorders in mammals, including impaired development of reproductive organs. However in human, isoflavones is associated with lower sperm concentration although unrelated to sperm motility and morphology or ejaculation volume.

Soybean has been well known to contain various nutrients function as antidiabetes, anticarcinogenic, antiproliferation, anticholesterol and hormone.[25,26] Chang *et al.*[27] reported that soybean has succeeded in controlling blood glucose level, lipid metabolism and anti-oxidant enzyme activity on type 2 DM. In addition, soybean is also beneficial in DM diet management. This study showed that ingestion of soybean powder suspension in diabetic rats increased serum testosterone level and expression of active caspase-3 Leydig cells, although the result did not reach statistical significance. Therefore, it can be concluded that ingestion of soybean powder suspension in diabetic rats does not impair their reproductive systems.

CONCLUSION

Soybean (*G. max*) powder suspension administration on diabetic rats which is induced by streptozotocin does not influence serum testosterone level and expression of active caspase-3 Leydig cells. In conclusion, soybean powder suspension does not deteriorate the reproductive system impairment of the diabetic rats.

ACKNOWLEDGEMENTS

Authors would like to thank the Director of Health Polytechnic, Department of Health, East Kalimantan, Drs. H Lamri, BS for his support in conducting this study. We would also like to thank Mrs. Yati, Mrs. Wiwid from Histology Laboratory, Mrs. Agustina from Department of Anatomy, as well as Mr. Soeparno from Department of Physiology, Faculty of Medicine, Universitas Gadjah Mada for their valuable assistance in conducting this study in laboratory.

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