Progress in Nutrition 2018; Vol. 20, Supplement 2: 257-262 DOI: 10.23751/pn.v20i2-S.5773

Goat-Soy Milk Kefir Increase Nitric Oxide Bioavailability by Increasing Endothelial Nitric Oxide Synthase (eNOS) Gene Expression in Diabetic rats

Alfi Muntafiah[1], Nurliyani[2], Sunarti[3]

[1]Department of Biochemistry, Faculty of Medicine, Universitas Jendral Soedirman, Purwokerto, Indonesia; [2]Department of Animal Product Technology, Faculty of Animal Science,Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia; [3]Department of Biochemistry, Faculty of Medicine, Public Health , and Nursing, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia - E-mail - nartyr@ugm.ac.id

Summary. *Objective:* Hyperglycemia-stimulated oxidative stress in diabetes mellitus impairs gene expression and activity of endothelial nitric oxide synthase (eNOS). Goat-soy milk kefir has potential to reduce hyperglycemia. This study is to investigate the benefit of kefir from goat and soy milk on eNOS gene expression and nitric oxide (NO) levels. *Methods:* Twenty five male Wistar rats were grouped as: 1) normal rats; 2) diabetic rats; 3) diabetic rats + goat milk kefir; 4) diabetic rats + combination of goat-soy milk kefir; 5) diabetic rats + soy milk kefir. After kefir intervention for 4 weeks, eNOS gene expression was determined using q-PCR and NO levels was analyzed by ELISA methods. *Results:* There were significantly differences of the NO levels among group after intervention of kefir for 4 weeks, and the highest NO level was found the diabetic rats with combination of goat-soy milk kefir ($p<0.05$). However, the eNOS gene expression was found highest in the diabetic rats with soy milk kefir. *Conclussion:* The combination of goat-soy milk kefir had good effects on expression of eNOS, NO levels and plasma glucose levels in diabetic rats.

Keywords: eNOS, gene expression, kefir, goat milk, soy milk.

Introduction

Diabetes mellitus is characterized by hyperglycemia, which induces oxidative stress through increasing reactive oxygen species (ROS) generation (1), which has a role in development of endothelial dysfunction (2). According to Hadi and Suwaidi (3), hyperglycemia is the major causal factor the endothelial dysfunction development that may be a consequence or cause of changing in NOS expression and activity. The abundance of ROS can inhibit endothelial nitric oxide synthase (eNOS) mRNA expression, and as an inhibitor to activity of nitric oxide (NO) (4). Reduction of eNOS expressions can lead decrease of NO bioavailability (5), that can as a risk factor for cardiovascular disease, one of the complications, in patients with type 2 diabetes mellitus (T2DM) (6). Therefore, controlling of hyperglycemia and oxidative stress are very important in T2DM patients.

Harmful effect of oxidative stress in cardiovascular function related with endothelial dysfunction lead to new development of pharmacotherapies strategy. Antioxidants have been reported can ameliorate hyperglycemia (7). Antioxidants remove free-radical by being oxidized themselves, and inhibit other oxidation reactions to stop the harmful chain reactions for all living cells. Nowadays, most food & pharmaceutical products contain synthetic antioxidants to enhance the stability of therapeutic agents. However, the synthetic antioxidants could have carcinogenic effects on human cells (8), so that natural antioxidants are the best choices. Several studies showed that fermented milks,

such as kefir, has radical scavenging activities, which can be used as natural antioxidant supplement for improving human health. Balakrishnan and Agrawal (9) reported that fermentation increases antioxidant activity of milks, and the increase in antioxidant activity of goat milk is highest compared to -cow or -camel milks after fermentation. Ostadrahimi et al. (10) and Nurliyani et al. (11) showed that kefir can improve glycemic control and increase glutathione peroxidase activity in T2DM. Kefir is a fermented milk product that can be made from cow's milk, goat's milk, sheep's milk, soy milk or other milk (12).

The previous studies showed that soy milk kefir has high ability to inhibit ascorbate autoxidation (13), reduce blood glucose levels, inflammatory marker levels, and increase number of Langerhans and beta cells in diabetic rats (14;11). Soy contains several nutrients such as isoflavones, protein, and phytoestrogens (15). Isoflavones are estrogen-like compounds that have estrogenic ability and antioxidant (12). Dajanta et al. (16) showed that fermentation process could increase antioxidant activity of soy milk. Therefore, in this study, we evaluate the effects of combination kefir of goat milk and soy milk on nitric oxide level and endothelial nitric oxide synthase (eNOS) gene expression on diabetic rats.

Materials and Methods

Soy milk preparation

Soy milk prparation was done according to Kasenkas et al.(13) with slight modification. Soy bean was washed and cleaned, then soaked in water for approximately 18 hours. After that, soy milk was separated from the soaking water and add water as much as 2 times the weight of soy milk that have been soaked. Then, it was blendered and filtered.

Kefir preparation

Kefir preparation was done according to Kasenkas et al. (13) with slight modification. Goat milk, soy milk, and combination of goat and soy milk (50% goat milk-50% soy milk) were pasteurized at 85°C for 10 min, then cooled at temperature. After that, each milk were inoculated by kefir grain as much as 2% and incubated at room temperature for 18h. At the end of fermentation process all fermented milk were stirred and filtered. Before used all of kefir were storage at 4°C.

Animal study

Twenty five (25) male Wistar rats (250-300 g, 3 months old) were obtained from the Faculty of Veterinary Medicine, Bogor Agricultural University (IPB). They were housed individually in cages and maintained under standard condition (22-25°C room temperature and 12-hour daylight cycle). They were acclimatized for 7 days by standard diet (AIN 93 M) (17) with slight modification. The diet consisted of cf corn starch 461g, 200g casein, corn oil 180g, DL-Methionine 3g, choline bitartat 1g, mineral mix 35g, vitamin mix 10g, to 50g, and sucrose 11 g (Percentages in diet composition means total% in 100g diet). This protocol was approved by The Ethics Committee for Health Research, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Induction of diabetes

Induction of diabetes was done according to Shirwaikar et al. (18). Diabetes was induced after overnight fasting by intraperitoneal injection of 60 mg/kg BW streptozotocin (STZ) (Nacalai, Tesque, Inc., Japan), 15 min after intraperitoneal injection of 120 mg/kg BW nicotinamide (NA) (Sigma-Aldrich, USA). Blood glucose was measured after 5 days of induction, and rats were considered as diabetic if they had fasting blood glucose levels ≥ 126 mg / dL.

Experimental study

Rats divided into 5 groups after induction of diabetes, each group consist of 5 rats : 1) normal rats; 2) diabetic rats; 3) diabetic rats with goat milk kefir; 4) diabetic rats with combination of goat-soy milk kefir; 5) diabetic rats with soy milk kefir. Kefir was given by gavage 2 mL/day for 4 weeks. Blood glucose were measured before and after kefir administration. At the end of the study, the blood was taken under anesthe-

sia to measure NO levels. Aorta tissues also taken to measure eNOS gene expressions. Tissues for RNA preparation were stored in freezer at -80°C after being snap-frozen liquid nitrogen.

Biochemichal analysis

Blood glucose was enzimatically analyzed using commercial kit (DiaSys, Germany). NO levels was analyzed by enzyme-linked immunosorbent assay (ELISA) method using commercial kit (R & D Elisa kits Systems' Total Nitric Oxide).

Isolation of RNA and quantitative polymerase chain reaction (q-PCR)

Total RNA was extracted from frozen aorta using Trizol reagent (Invitogren, USA). Reverese transcriptation was done according to iScript cDNA synthesis kit (Bio-rad, UK) protocol. The q-PCR was used by SsoFastEvaGreenSupermix (Bio-rad, United Kingdom) and total reaction for q-PCR was 10µL. Specific primers is that eNOS forward: 5'- TGT CAC CAT GGA AAG ACA AG -3 ', eNOS reverse: 5'- ACC AAG TGG ATC TCT TGA AC -3' (223 bp), β-actin sense: 5 '- AAC AAC CGT CCT GAA GCC AAG AAG-3 'and β-actin antisense: 5'-TCA TGA CTG AGT GGT GGT TCA-3' (241 bp). Detection of eNOS expression was done by running with Bio-Rad CFX96TM real-time PCR system. The protocol starts with an initial denaturation at 95 °C for 30 seconds, then 40 thermal cycles of denaturation at 95 °C for 5 seconds, annealing and extension at 60 °C for 5 seconds, and followed by the last one cycle of melting curve at a temperature of 65-95 °C increments of 0.5 °C for 5 sec / step.

Statistical analysis

All values were presented as mean ± standard deviation (SD). One-way ANOVA was used to analyzed the differences of plasma glucose levels, NO levels, and eNOS gene expression between the groups. Differences were considered statistically significant at $p<0.05$.

Results

Plasma glucose levels

After induction of diabetes, plasma glucose levels were similiar among diabetic control group and treatment groups. After 4 weeks administration of kefir, plasma glucose levels in all treatment groups significantly reduced compared in diabetic control group ($p=0.006$). Diabetic rats with combination of goat-soy milk kefir has lower plasma glucose levels comapared diabetic rats with goat milk kefir and soy milk kefir (Table 1).

Nitric oxide levels

After kefir administration for 4 weeks, NO levels in all treatment groups were significantly higher than

Table 1. Plasma glucose levels before and after kefir administration

Groups	Plasma glucose level (mg/dL)			
	Before	After	Mean difference	p
Normal	73.21 ± 1.19[a]	116.36 ± 1.0[s]a	43.14	0.001
Diabetic	258.34 ± 1.60[b]	371.79 ± 1.26[b]	113.45	0.090
Diabetic + goat milk kefir	208.55 ± 1.42[b]	124.17 ± 1.29[a]	-84.38	0.043
Diabetic + combination of goat -soy milk kefir	336.36 ± 1.83[b]	112.00 ± 1.11[a]	-224.36	<0.001
Diabetic + soy milk kefir	297.17 ± 1.0[s]b	226.05 ± 2.31[a,b]	-72.81	0.456
p	<0.001	<0.001		

Values are presented as mean ± standard deviation (SD), n=5. [a nad b] Indicate p<0.05 according to One-way ANOVA test, followed by Games-Howell test. [a,b] Indicate no difference either [a] nor [b].

diabetic control group. Diabetic rats with combination of goat-soy milk kefir has the highest NO levels than diabetic rats with goat milk kefir or soy milk kefir (Figure 1).

eNOS gene expressions

Analysis of eNOS gene expression showed that the administration of goat kefir did not influence the eNOS gene expression, but it was influenced by soy kefir. Therefore, the highest eNOS gene expression was found in diabetic rats with soy kefir **(Figure 2).**

Discussion

This study showed that the diabetic rats have plasma NO levels lower than normal rats (Table 2). The low NO levels in diabetic condition may be caused by oxidative stress that is induced by hyperglycemia (Table 1). NO is a gaseous lipophilic free radical, which is produced by three different isoforms of NOS; neuronal NOS (type 1 or nNOS), inducible NOS (type 2 or iNOS), and endothelial NOS (type 3 or eNOS) (19). According to Giacco and Brownlee (1), hyperglycemia increases production of reactive oxygen species (ROS).

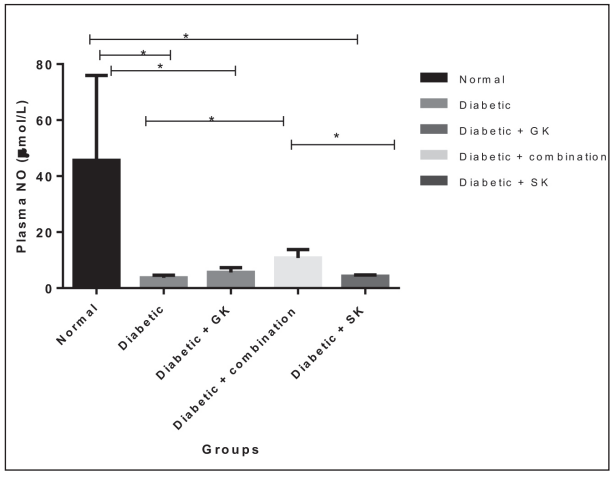

Figure 1. Plasma NO levels (µmol/L) after kefir administration for 4 weeks according to One-way ANOVA test followed by Games-Howell test. Normal: non diabetic rats (35.24 ± 2.47); Diabetic rats (3.64 ± 1.23); Diabetic+GK: diabetic rats received goat milk kefir (5.49 ± 1.32); Diabetic combination: diabetic rats received combination of goat-soy milk kefir (10.34 ± 1.37); Diabetic SK: diabetic rats received soy milk kefir (4.21 ± 1.11).

Superoxide is a ROS when reacting with NO produces peroxynitrite that induces cellular damage through cofactor depletion of eNOS (tetrahydrobiopterin) (19) that cause reducing NO production in diabetes. The results of this study are consistent with the theory that showed the diabetic rats have low levels of NO. Beside as a result of low activity of eNOS, the low NO levels can be caused also by changing of endothelial eNOS. Endothelial eNOS is the major source of NO in the vasculature, and expression of eNOS is reported undergo alteration diabetes mellitus (19). Our results also supported by Felaco et al.(20) that showed diabetic rats had lower eNOS gene expressions, which contributes decreased NO production (21). The report is consistent with the result in this study that diabetic rats had low eNOS expression and NO levels.

\Your study result should supported by the results of other researchs

The NO levels in diabetic rats with combination of goat-soy milk kefir were higher than those in diabetic rats with goat milk kefir or with soy milk kefir, although the eNOS gene expression was found highest in the diabetic rats with soy milk kefir. High NO level in diabetic rats with combination of goat -soy milk kefir may be caused by components in the goat and soy that can reduce oxidative stress. According to Matsunami et al. (22) superoxide dismutase (SOD) activity decreased significantly in the erythrocytes and all organs of diabetic rats, and goat milk has positive

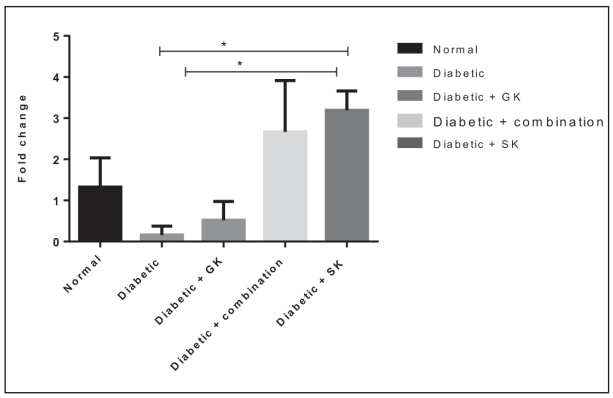

Figure 2. eNOS gene expression after 4 weeks kefir administration. p<0.05 according to One-way ANOVA test followed by Games-Howell test. Diabetic + GK = diabetic + goat milk kefir; Diabetic + Combination = diabetic + combination of goat-soy milk kefir; Diabetic + SK = diabetic + soy milk kefir.

effects on antioxidant defence through increased activity of SOD (23). Fermentation process increases antioxidant activity of the milks, that were 93 % of fermented goat milk, 86 % of camel milk and 79 % of cow milk (9).

In addition, soybean has been reported preventing some abnormal changes in vascular reactivity in diabetic rats through nitric oxide- and prostaglandin-related pathways and via attenuation of oxidative stress in aortic tissue and endothelium integrity (24). According to Kwon et al. (15), soy contains several nutrients such as isoflavones, protein, and phytoestrogens. Dietary isoflavones have ability to activate signaling pathways leading to increase NO bioavailability and antioxidant enzyme expression (25;26) and induced increases in eNOS gene expression lead to improved endothelial function and reduced blood pressure in vivo (26). Soy isoflavone can increase endothelial nitric oxide synthase and decrease oxidative stress in ovariectomized rats (27).

Conclusions

Is this paragraph a coclusion paragraph?

In this study showed that the effects of goat milk kefir on plasma glucose and NO levels was slightly higher than soy milk kefir, but soy milk kefir increased significantly the expression of eNOS. However, the combination of goat-soy milk kefir had good effects on expression of eNOS, NO and plasma glucose levels. According to Hadi and Suwaidi (3) and Silva et al. (2) the hyperglycemia that is the major causal factor the endothelial dysfunction development, which can be related with the changing in expression and activity of eNOS. Hyperglycemia increase ROS production (1), that can inhibit endothelial nitric oxide synthase (eNOS) mRNA expression, and as an inhibitor to activity of nitric oxide (NO) (4). Therefore, reduction of eNOS expressions can lead decrease of NO bioavailability (5), that can as a risk factor for cardiovascular disease, one of the complications, in patients with T2DM (6). In conclusion, administration of combination of goat-soy milk kefir increase effectively eNOS expression and NO bioavailability in diabetic rats. Based on this study we suggested the type 2 diabetic patients consume the combination of goat-soy milk kefir to increase bioavailability of NO.Suggestions should be added

Acknowledgement

This study was supported by grant from Public Fund (Dana Masyarakat) Faculty of Medicine Gadjah Mada University (Yogyakarta). This paper is part of author's (Alfi Muntafiah) thesis.

References

1. Giacco F and Brownlee M. Oxidative stress and diabetic complications. Circ Res 2010; 107(9): 1058–1070.
2. Silva BR, Pernomian L, Bendhack LM. Contribution of oxidative stress to endothelial dysfunction in hypertension. Front Physiol 2012; 3 (441): 1-5.
3. Hadi HAR and Suwaidi JA. Endothelial dysfunction in diabetes mellitus. Vasc Health Risk Manag 2007; 3(6): 853–876.
4. Higashi Y, Maruhashi T, Noma K, Kihara Y. Oxidative stress and endothelial dysfunction: clinical evidence and therapeutic implications. Trends Cardiovasc Med 2014; 24(4):165-169.
5. Sena CM, Pereira AM, Seiça R. Endothelial dysfunction — a major mediator of diabetic vascular disease. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 2013; 1832 (12): 2216-2231.
6. Creager MA, Lüscher TF, Cosentino F, Beckman JA. Diabetes and vascular disease pathophysiology, clinical consequences, and medical therapy: Part I. Circulation 2003; 108 (12): 1527-1532.
7. Wang F, Pu C, Zhou P, Wang P, Liang D, Wang Q, Hu Y, Li B, Hao X. Cinnamaldehyde prevents endothelial dysfunction induced by high glucose by activating Nrf2 2015; 36(1):315-324.
8. Shebis Y, Iluz D, Kinel-Tahan Y, Dubinsky Z, Yehoshua Y. Natural antioxidants: function and sources. Food and Nutrition Sciences 2013; (4): 643-649.
9. Balakrishnan, G, and Agrawal, R. Antioxidant activity and fatty acid profile of fermented milk prepared by Pediococcus pentosaceus. J Food Sci Technol 2014; 51 (12):4138–4142.
10. Ostadrahimi A, Taghizadeh A, Mobasseri M, Farrin N, Payahoo L, Beyramalipoor Gheshlaghi Z, Vahedjabbari M. Effect of probiotic fermented milk (kefir) on glycemic control and lipid profile in type 2 diabetic patients: a randomized double-blind placebo-controlled clinical trial. Iran J Public Health 2015; 44(2):228-237.
11. Nurliyani, Harmayani E, and Sunarti. Antidiabetic potential of kefir combination from goat milk and soy milk in rats in-

duced with streptozotocin-nicotinamide. Korean J Food Sci Anim Resour 2015; 35(6): 847–858.
12. Liu JR, Chen MJ, Lin CW. Antimutagenic and antioxidant properties of milk-kefir and soymilk-kefir. J Agric Food Chem 2005; 53(7):2467-2474.
13. KesenkaşH., Dinkci N., Seckin K, Kinik O, Gonc S. Antioxidant properties of kefir produced from different cow and soybean milk mixtures. J Agric Sci 2011; 17:253-259.
14. Sunarti, Nurliyani, Tyas ASA, Krstian SD, Prasetyastuti. The influence of goat milk and soybean milk kefir on il-6 and crp levels in diabetic rats 2015; 22 (3): 261-267.
15. Kwon DY, Daily JW 3rd, Kim HJ, Park S. 2010. Antidiabetic effects of fermented soybean products on type 2 diabetes. Nutr Res 2010; 30(1):1-13.
16. Dajanta K, Janpum P, Leksing W. Antioxidant capacities, total phenolics and flavonoids in black and yellow soybeans fermented by Bacillus subtilis: a comparative study of thai fermented soybeans (thua nao). International Food Research Journal 2013; 20(6): 3125-3132.
17. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993; 123(11):1939-1951.
18. Shirwaikar A, Rajendran K, Barik R. Effect of aqueous bark extract of Garuga pinnata Roxb. in streptozotocin-nicotinamide induced type-II diabetes mellitus. J Ethnopharmacol 2006; 107(2):285-90.
19. Pitocco D, Zaccardi F, Di Stasio E, Romitelli F, Santini SA, Zuppi C, Ghirlanda G. 2010. Oxidative stress, nitric oxide, and diabetes. Rev Diabet Stud 2010; 7(1):15-25.
20. Felaco M, Grilli A, De Lutiis MA, Patruno A, Libertini N, Taccardi AA, Di Napoli P, Di Giulio C, Barbacane R, Conti P. Endothelial nitric oxide synthase (eNOS) expression and localization in healthy and diabetic rat hearts. Ann Clin Lab Sci 2001; 31(2):179-86.
21. Sena CM, Nunes E, Louro T, Proença T, Fernandes R, Boarder MR, Seiça RM. Effects of alpha-lipoic acid on endothelial function in aged diabetic and high-fat fed rats. Br J Pharmacol 2008 ;153(5):894-906.
22. Matsunami T, Sato Y, Sato T, Yukawa M. Antioxidant status and lipid peroxidation in diabetic rats under hyperbaric oxygen exposure. Physiol Res 2010; 59(1):97-104.
23. Dı´az-Castro J, Pe´rez-Sa´nchez LJ, Lo´pez-Frı´as MR, Lo´pez-Aliaga I, Nestares T, Alfe´rez MJM, Ojeda ML and Campos MS. Influence of cow or goat milk consumption on antioxidant defence and lipid peroxidation during chronic iron repletion. Br J Nutr 2012; 108(1):1-8.
24. Baluchnejadmojarad T, Roghani M, Dehkordi FR. Soybean feeding improves vascular dysfunction and attenuates oxidative stress in streptozotocin-diabetic rats 2012; JBCP 1 (1): 37-45.
25. Siow RC, Mann GE. Dietary isoflavones and vascular protection: activation of cellular antioxidant defenses by SERMs or hormesis?. Mol Aspects Med 2010; 31(6):468-77.
26. Mahn K, Borrás C, Knock GA, Taylor P, Khan IY, Sugden D, Poston L, Ward JP, Sharpe RM, Viña J, Aaronson PI, Mann GE. Dietary soy isoflavone induced increases in antioxidant and eNOS gene expression lead to improved endothelial function and reduced blood pressure in vivo. FASEB J 2005; 19(12):1755-7.
27. Tang Y, Li S, Zhang P, Zhu J, Meng G, Xie L, Yu Y, Ji Y, and HanY. Soy isoflavone protects myocardial ischemia/reperfusion injury through increasing endothelial nitric oxide synthase and decreasing oxidative stress in ovariectomized rats. Oxid Med Cell Longev 2016: 1-14.

Correspondence:
Sunarti
Department of Biochemistry, Faculty of Medicine, Universitas Gadjah Mada
Yogyakarta, Indonesia 55281
Tel. +628112542918
E-mail - nartyr@ugm.ac.id