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ORIGINAL ARTICLE

The Effect of Folate Administration on Serum Homocysteine Level and Diabetic Retinopathy Parameters an Experimental Study on Sprague-Dawley rats

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ABSTRACT

Background: Hyperhomocysteinemia has been associated with an increased risk of diabetes complications, such as diabetic retinopathy(DR) which can lead to blindness. Hyperglycemia stimulates retinal oxidative stress and increases nitric oxide(NO), that affects to the retinal blood vessels (RBV)and DR. Folic acid (FA) administration decreasesserum Hcy.
Aim: To analyze the effect of folate on Hcy level and DR parameters(retinal VEGF expression, quantity of new RBV and RBV leakage).
Methodology: An experimental study using a randomized controlled group pretest posttest design.Forty male Sprague-Dawley rats were divided into 5 groups: Negative, positive control (Streptozotocin40mg/kg BW induced) , Streptozotocin 40mg+ FA 2, 4, 8 ppm for 30 days). Serum Hcy and DR parameters were measured before and after intervention. Differences of groups were measured with Wilcoxon test.
Result: Two and 4 ppm FA didn't decrease serum Hcy level and DR parameters (p>0.05); while 8 ppm FA showed asignificant decreased serum Hcy(p0.043) and DR parameter which are VEGF expression (p0.038), quantity of new RBV (p0.034) and RBV leakage (p0.041).The quantity of new RBV was increased in the positive control(p 0.041) as well as RBV leakage quantityin the positive control(p0.041) and 2 ppm FA group (p0.042).
Conclusion: Administration of 2 and 4 ppm FA didn't decrease serum Hcy and DR parameters but the 8 ppm FA significantly decreasedserum Hcy levels and DR parameters.
Keywords: Homocysteine, diabetic retinopathy parameters

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by an increase in blood glucose level as a result of defects in insulin secretion, insulin action or both. Increasing prevalence of diabetes has been reported due to population growth, aging, urbanization, increasing prevalence of obesity and physical inactivity.Hyperglycemia in diabetes can cause complications such as dysfunction and failure of various organs; especially the eyes, kidneys, nerves, heart and blood vessels[1].

Diabetic retinopathy(DR) is a common complication of diabetes, which is also the leading cause of low vision and blindness worldwide. The effective treatment has not yet been found;consequently finding new risk factors and biomarkers to prevent progression of DR is important. Several known risk factors associated with the incidence of DR are blood pressure, glycemic status, blood urea nitrogen, creatinine and beta 2 microglobulin. However,there were many other aspects of DR's risk factorsthat should be considered.[2]

Homocysteine (Hcy) is a highly reactive thiol containing amino acid derived from the conversion of methionine to cysteine, which produces reactive oxygen species –hydrogen peroxide and superoxide anion radical. Elevated plasma Hcy level is vasculotoxic as it causes endothelial dysfunction, increased oxidative stress, altered coagulation/fibrinolysis, smooth muscle cell proliferation and changes in structural and elastic properties of the vessel wall. It also limits nitric oxide(NO) production and promotes lipid peroxidation, thereby decreasing the bioavailability of NO.Pro-oxidative state in diabetes isworsenedby auto-oxidation of Hcy, leading to additional oxidative stress and thereby to endothelial dysfunction, platelet activation and thrombus formation.[2,3] Endothelial dysfunction is characterized by impaired endothelium-dependent vaso-relaxation due tothe loss of NO bioactivity in blood vessel walls[4].

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Brazionis study suggests homocysteine is associated with diabetic retinopathy in type 2 diabetes, independent of the major determinants of both retinopathy and homocysteine levels, and that homocysteine concentrations would be higher in diabetic individuals withretinopathy than in those without retinopathy[5]. Hyperhomocysteinemia is known to be a risk factor for vascular occlusive diseases. Elevated levels of plasma homocysteine have been found in patients suffering from peripheral vascular occlusions, such as coronary artery disease, cerebral vascular accidents and deep-vein thrombosis, as well as from ocular vascular occlusions, such as retinal vein and retinal artery and anterior ischemic optic neuropathy[6].

Mild hyperhomocysteinemia (HHcy) is defined as a condition where plasma Hcy level isabove15 µmol/L and will alter oxidative stress induction and endothelial cell arginine transport inhibition. However, the role of mild HHcy and oxidative stress,along withits correlation with development of DR are still unknown and requires further investigation. Although many studies have evaluated the association between Hcy and DR, the results are varied and inconsistent. Various studies have proposed that mild HHcy and oxidative stress mightplay role in development of DR; in contrast, some other studies suggested no significant correlation between Hcy and DR.[7]Several studieshave reported correlation between plasma Hcy level and DR prevalence. Total plasma Hcy level may becomea useful biomarker to assess increasing risk of DR in people with type 2 DM[8].

Diabetic retinopathy is a major cause of new onset blindness among diabetic adults and characterized by increasing vascular permeability, tissue ischemia and neovascularization. Neovascularization of retina carries high risk of blindness as a result of vitreous hemorrhage and fibrosis. Vascular endothelial growth factor(VEGF) can stimulate angiogenesis, enhance collateral vessel formation and increase the permeability of the microvasculature. In DR, VEGF plays a role in the neovascularization of proliferative retinopathy and in blood retinal vessels breakdown and the levels was markedly elevated in the vitreous and aqueous fluids.[9]Diabetic retinopathy in rat can be assessed with increasing level of plasma VEGF and its expression in retina. Hyperglycemia spur of VEGF expression due to oxidative stress induced by HHcy, will lead to PKC activation, NO increment, VEGF and NO uncoupling, impaired regulation of retinal blood vessels and DR[10,11].

Folate and vitamin B12 belong to the group of water-soluble B vitamins that occurs naturally in food.green leaf vegetables (such as spinach and turnip greens), fruits (such as citrus fruits and juices) and dried beans and peas are all natural sources of folate. Folic acid is the synthetic form of folate found in supplements and added to fortified foods. Folate functions as a coenzyme in single-carbon transfers in the metabolism of nucleic and amino acids, and therefore is especially important during periods of rapid cell division and growth such as during infancy and gestation.[12-14]Nutritional deficiencies, particularly those involving B-group vitamins and folate (which are important cofactors of Hcy metabolism), are commonly related with high circulating levels of Hcy. Therefore, changes in folate status may influence DNA stability and integrity and also affect methylation patterns in neural tube tissue which could predisposeto the development of DR. However, very little evidence is currently available to suggest that folate deficiency alone leads to DR[15].

MATERIALS AND METHODS

This was an experimental study using a randomized controlled group pre-test and post-test design, conducted in LPPT (Integrated Research and Testing Laboratory) units IV Gadjah Mada University Yogyakarta, Pathology anatomyDepartment (Faculty of Medicine Gadjah Mada University and Prof. Dr. Sardjito Hospital, Yogyakarta) and IDD Laboratory (Faculty of Medicine, Diponegoro University, Semarang) from July to December 2010. Sprague-Dawley rats aged 3-4 months were purchased from LPPT. Sample size was determined at 5 animals for each group, based on WHO manual and Federer formula. Inclusion criteria were: body weight (BW) 190-275 g, healthy, active, not disabled, initial (screening) fasting blood glucose (FBG) levels should be less than110 mg/dl. This study consist of 5 group which are: negative control, positive control (40mg/kgBW streptozotocin/STZ intra peritoneal induction), X1 (STZ + folic acid/FA 2 ppm administration through nasogastric tube for 30 days), X2 (STZ + FA 4 ppm), X3 (STZ + FA 8 ppm).FBG levels post-inductionshould be more than 200mg/dl. Levels of blood glucose were examined using Acucheck, serum Hcywith Enzyme Linked Immunosorbent Assay(ELISA) method[16-18].

Retinal VEGF expression was examined by immunohistochemistry (IHC) staining using light microscope Nikon Eclipsse E600W #725246 with Nikon camera Dn 100.Immunostaining degree was divided to: minimal (almost no stained retina), moderate (red, light brown in the retina), severe (red, strong dark brown).New retinal blood vessels werequantified withhematoxyllin-eosin(HE) staining based on the number of retinal blood vessel cells nuclei,and would represent the growth of new

retinalblood vessels when located on the vitreous inner limiting membrane side. Evaluated on five field of view, calculated the number of nuclei of retinal vessel cells. [19-22]Quantity of retinal blood vessels leakage was examined using retinal fluorescein angiography with high molecular weight fluorescein isothyocyanate dextran (FITC) MW 2 million, it dissolved in PBS (phosphate buffer saline) to 1 ml volume[19-22]. Rats were anesthetized with ether and then injected using tuberculin syringe(iv) on the tail. Two hours later the eye was enucleated and soaked on a 4% paraformaldehyde / PA solution (Sigma Chemical Co) for 30 minutes. Anterior Segment, Lens and vitreous removed, retina immersed in PA Solution for 3-hour. Posterior eye cup made 6 radial pieces on each quadrant then placed on the gelatin – coated slide, flat mounted position and sealed with transparent nail polish. Leakage of fluorescein solution indicates leakage of retinal blood vessels. Scoring of retinal blood vessels was performed by fluorescein microscope.

All microscopic evaluation done by two examiners at 5 different field of view, the suitability of the results between the two was analyzed by Spearman correlation test and different test with Wilcoxon test.Differenceof all parameters before and after intervention were analyzed by using SPSS 11.5for Wilcoxon test. [23]

Ethical clearance of research was obtained through the Health & Medical Research Ethics Commission of Faculty of Medicine, Diponegoro University and Kariadi Hospital Semarang Indonesia no 49/EC/FK//RSDK/2010 dated June 10, 2010

RESULTS

Fasting blood glucose(FBG)levels: The mean fasting blood glucose screening levels were less than 110 mg/dl which increased to above200 mg/dl withinthree weeks after induction of STZ, and remained at elevated levels after intervention, with the highest level observed in group of 2 ppm FA (Table 1).

Homocysteinelevel: Administration of 2 and 4 ppm FA didn't significantly decrease serum Hcy level (p>0,005),while administration of 8 ppm FA significantly decreased serum Hcy level (p= 0.043), we can see in table 2.

Administration of 8 ppm FA significantly decreased retinal VEGF expression (p= 0.038) (table 3)

Administration of 8 ppm FA significantly decreased the quantity of new retinal blood vessels (p=0.034),while quantity of new retinal blood vessels increased significantly in the positive control group (p= 0.041)(table 4).

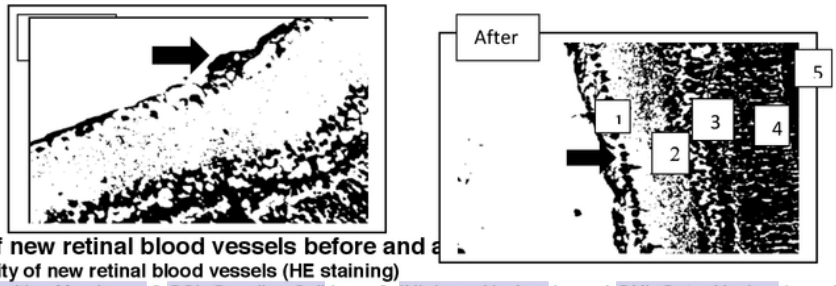
Administration of 8 ppm FA significantly decreased the quantity of retinal blood vessels leakage (p = 0.041) (table 5). On the contrary,quantity of retinal blood vessels leakage in the positive control group (p=0.041) and FA 2ppm (p=0.042) were significantly increased.

Retinal VEGF expression (IHC staining)

Fig 1: Retinal VEGF expression (IHC staining) before and after intervention

1. ILM:Inner Limiting Membrane 2. GCL:Ganglion Cell Layer3.INL:Inner Nuclear Layer4. ONL:Outer Nuclear Layer5.OLM:Outer Limiting Membrane6.RPE:Retinal Pigment Epithelium

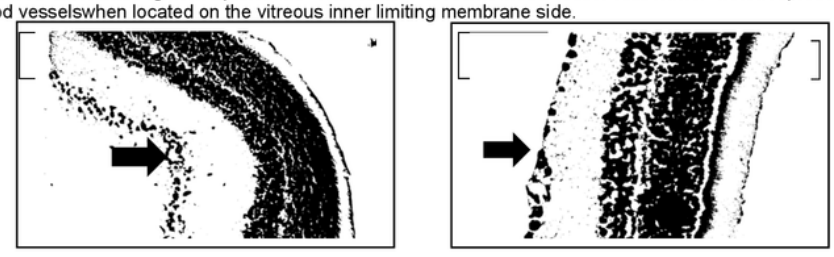
Immunostainingdegree was divided to: minimal (almost no stained retina), moderate (red, light brown in the retina), severe (red, strong dark brown).



Quantity of new retinal blood vessels before and a

Fig 2: **Quantity of new retinal blood vessels (HE staining)**

1. ILM:Inner Limiting Membrane 2.GCL:Ganglion Cell Layer3. INL:Inner Nuclear Layer4.ONL:Outer Nuclear Layer5.OLM:Outer Limiting Membrane6. RPE:Retinal Pigment Epithelium. The number of retinal blood vessel cells nuclei would represent the growth of new retinalblood vesselswhen located on the vitreous inner limiting membrane side.



Quantity of retinal blood vessels leakage before and after intervention

Fig. 3: Retinal blood vessels leakage before and after intervention (Retinal fluorescein angiography)
Leakage of fluorescein solution indicates leakage of retinal blood vessels . Scoring of retinal blood vessels was performed by fluorescein microscope.

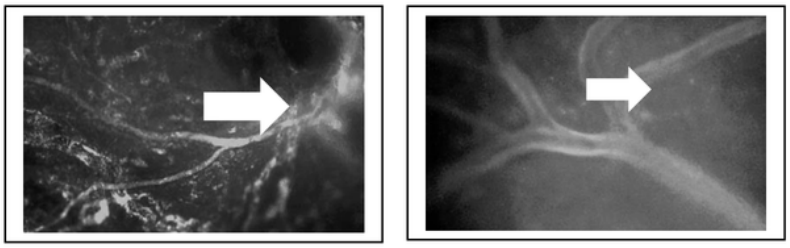


Table 1: Mean fasting blood glucose (FBG) levels before and after intervention

	Screening (mg/dl)			Before intervention (mg/dl)			After intervention (mg/dl)		
Negative control	71.2	±	8.47	70.5	±	5.24	84.3	±	9.69
Positive control	79.2	±	6.53	287.4	±	42.37	443.0	±	122.56
FA 2ppm	75.8	±	9.78	256.0	±	83.69	463.8	±	116.77
FA 4 ppm	73.0	±	3.87	357.0	±	55.07	362.8	±	76.93
FA 8 ppm	81.4	±	5.63	264.0	±	29.25	373.0	±	126.74

Table 2:Homocysteine level before and after intervention.

Group	Mean ± SD						p
	Before intervention			After intervention			
Hcy level (µmol/L)							
Negative control	20.5	±	4.37	21.2	±	2.78	0.456
Positive control	27.4	±	5.32	27.2	±	12.1	0.785
FA 2 ppm	24.2	±	3.43	22.3	±	14.06	0.344
FA 4 ppm	28.6	±	4.83	17.8	±	4.66	0.080
FA 8 ppm	25.8	±	8.93	15.0	±	1.58	0.043

Table 3: Retinal VEGF expression differences before and after intervention (IHC staining)

Group	VEGF Expression										p
	Before intervention					After intervention					
	FV1	FV2	FV3	FV4	FV5	FV1	FV2	FV3	FV4	FV5	
Negative control	min	min	min	min	min	min	min	min	min	min	1.00
Positive control	mod	mod	sev	sev	Sev	mod	mod	mod	sev	sev	0.317
FA 2ppm	sev	mod	sev	sev	Mod	sev	mod	mod	mod	mod	0.157
FA 4 ppm	sev	sev	sev	mod	Sev	mod	mod	mod	mod	sev	0.083
FA 8 ppm	sev	sev	mod	sev	Sev	min	min	min	mod	min	0.038

Note: FV : Field of view mod : moderate min : minimal sev : severe

Table 4: Quantity of new retinal blood vessels before and after intervention (Hematoxillin-eosin staining)

Group	Quantity of blood vessels nuclei										p
	Before intervention					After intervention					
	FV1	FV2	FV3	FV4	FV5	FV1	FV2	FV3	FV4	FV5	
Negative control	0	0	0	0	0	0	0	0	0	0	0.317
Positive control	7	8	7	6	7	15	17	16	17	15	0.041
FA 2ppm	8	7	8	7	8	6	6	5	7	6	0.066
FA 4 ppm	7	6	6	7	7	6	5	5	7	7	0.083
FA 8 ppm	8	8	7	8	9	3	3	4	3	4	0.034

Note: FV :Field of view

Table 5: Quantity of retinal blood vessels leakage before and after intervention.(Retinal fluoresceine angiography)

Group	Quantity of retinal blood vessels leakage										p
	Before intervention					After intervention					
	FV1	FV2	FV3	FV4	FV5	FV1	FV2	FV3	FV4	FV5	
Negative control	0	0	0	0	0	1	0	0	0	0	0.317
Positive control	3	3	4	2	3	8	6	7	8	8	0.041
FA 2ppm	3	3	2	3	2	5	6	7	8	3	0.042
FA 4 ppm	4	9	5	3	4	3	4	4	3	4	0.157
FA 8 ppm	5	4	5	5	5	2	1	1	0	2	0.041

Note: FV :Field of view

DISCUSSION

Increased weight of negative control rats post intervention suggests that rats were well treated. Blood glucose levels of all rats increased, the highest was observed in FA 2 ppm group. Chronic hyperglycemia contributionto the development of diabetic retinopathy (DR) has been reported, which is why good glycemic control becamethe best choice to inhibit or to prevent the development of diabetic retinopathy. In this study, the FA 2 ppm group experienced the smallest decrease of serum Hcy level and highest blood glucose level post intervention;whileserumHcy level decreased significantly in FA 8ppm group (p= 0.043).

Folic acid is the most important dietary determinant of homocysteine; daily supplementation of0.5 to 5.0 mg typically lowers plasma homocysteine levels by about 25%[24]. Atta(2005 and 2007) found similar result,whereby FA decreased serum Hcy and VEGF levels[25,26]. Satyanarayana (2011) stated that Hcy is negatively correlated with FA.[27] Recent studies reported that FA play a role in Hcy metabolism and interact with NO, which cause an increase of tetrahydrobiopterin availability, reduce the formation of superoxide anion and improve endothelial dysfunction (Diez, 2005)[28]. High-dose FA is required to reduce Hcy level. This study result was in linewith (HOPE) 2 Investigators, Atta, Satyanarayana and confirmed finding ofDiez[24,26,27,28]. The result of this study was different to Nieman (2006)[29]. Total Hcy level in rats that were not given FA increased by 4-5 times compared to rats that were given FA 2 and 8 ppm. However, there was no difference between the two groups (FA 2 and 8 ppm). This study concluded that high dose of FA is needed to decreaseserum Hcy level. The homocysteine lowering Trialist's Collaboration meta-analysis found that FA of 0.5 to 5 mg/day decreasedHcylevel by 25%[30].

The interaction between DM and increased Hcy in vascular complications were allegedly caused by oxidative stress, endothelial damage, and decreased bioavailability of nitric oxide (NO).[31]It has been reported that retinal blood flow changes in patients with DR. Because NO is thought to play an important role in the regulation of retinal blood flow, future investigations of the relationship between NO and retinal blood flow may be needed to understand the pathogenesis of DR.[32]

Wilmink (2000) stated that FA consumed orally could improve endothelial dysfunction and eliminate the increase of radical damage end products induced by triglyceride-rich lipoproteins.[33]Title study (2006) reported that improvement of endothelial dysfunction in type 2 diabetes without vascular disorders are independent of Hcy decrease[34].

Administration FA of 8 ppm significantly reduced retinal VEGF expression (p= 0.038). This result was in line with Hammes who reported that STZ induction could increase retinal VEGF expression. This study also confirmed Atta's study on decreased expression of VEGF in the provision of new high-dose FA. Retinal VEGF expression is associated with development of new blood vessels growth in the rat model of PDR. Zhang (2004) stated thatVEGF expression was detected in the cornea, iris, retina, choroid retinal pigment epithelial complex, ciliary body, the walls of blood vessels, and ocular muscle.[35]In the retina, VEGF staining was predominant in the inner limiting membrane, ganglion cell layer and inner nuclear layer. Weak positive staining for VEGF was also found in the outer limiting membrane and retinal pigment endothelium. Pierce (1995) reported that retinal VEGF expression was increased significantly in diabetic rat compared to control animal.[36] Retinal VEGF expression was found prior to the development of neovascularization in a mouse model of proliferative retinopathy. The VEGF level increased dramatically within 6-12 hour following relative retinal hypoxia and remains elevated until neovascularizations developed.

This study found that quantity of new retinal blood vessels increased significantly within positive control group (p =0.041), which indicate development of PDR onthe rats. This study isindirectly in linewith Hammes' study who found an increase of retinal VEGF expression on STZ induced diabetic rats .[37] This study is also in agreement with Atta's study that observed a significant retinal VEGF expression reduction with administration high dose FA (8ppm), where high dose of FA inhibits or reduces the quantity of the new blood vessels growth[26]. Diabetic

rats that were not injected with folate experienced a significant increase of retinal blood vessels quantity (experiencingPDR). Folateis not indicated to reduce BG level, as was shown from the increased level in results of post-treatment negative and positive control group. Folate decreases Hcy serum, which was proved by the high Hcy level and significaant increase of new retinal blood vessels quantity on positive control group that were not injected with folate.Hence, further research is required.

The quantity of retinal blood vessels leakage increased significantly in the positive control group, due to longer duration of DM without any treatment received. Similar result occured in 2ppm FA group. Injection of 2 ppm FA could not prevent an increase in the quantity of retinal blood vessel leakage. In this study, rat with high serum Hcylevel (positive control group) also experienced the most severe retinal blood vessel leakage comparedwith other groups. Injection of 8ppm FA caused a significant decrease in the quantity of retinal blood vessel leakage. Long duration of diabetes is associated with changes in retinal blood vessels, progressive loss of retinal capillaries that lead to retinal ischemia, increase growth factor and abnormal proliferation of new blood vessels. Retinal blood vessels are fragile andvulnerable to bleeding, scarring and fibrosis. VEGF plays a role in the growth of new blood vessels at the PDR and the opening of blood retinal barrier which is typically characterized by retinal blood vessel leakage,all these had been proven bythis study.

One of diabetic retinopathy diagnosis technique is by using retinal fluorescein angiography. Typically, fluorescein solution would be able to pass through tight junctions of retinal capillaries. However, in diabetic retinopathy, fluorescein fluid is more likely to leak. This method is useful for early detection of changes in blood retinal barrier, capillary closure and microaneurysms formation. This study was conducted using SD rats, in which retinal fluorescein angiography was performedwith a solution of high molecular weight fluorescein isothyocyanat dextran MW 2 million 50 mg/ml injected intravenously within 2 hours sequence. This solution has been often used for studies on oxygen-induced retinopathy rat model but has never been used for studies ofdiabetic retinopathy.

CONCLUSIONS

Administration of 2 and 4 ppm FA didn't decrease serum Hcy level and DR parameters. Administration of 8 ppm FA significantly decreased serum Hcylevel and the parameters of diabetic retinopathy (retinal VEGF expression, quantity of new retinal blood vessel and retinal blood vessel leakage)

Further studies in humans are expected to prevent and/or reduce the incidence of diabetic retinopathy. Retinal VEGF expression, the quantity of new retinal blood vessels and leakage of retinal blood vessels can be used to describe diabetic retinopathy in rats.

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