Total Plasmatic Homocysteine and von Willebrand Factor in Experimental Diabetes Mellitus

Renato Delascio Lopes, Lindalva Batista Neves, Vânia D'Almeida, Gleice Margarete de Souza Conceição, Alexandre Gabriel Junior

Universidade Federal de São Paulo – UNIFESP-EPM - Sao Paulo, SP, Brazil

Summary

Objectives: To determine the plasma homocysteine and von Willebrand factor levels as markers of endothelial dysfunction in rats with diabetes mellitus induced by streptozotocin.

Methods: Thirty-five adult male rats (Rattus norvegicus albinus) (weight between 180-200g) were randomized into three groups: control group (n=10), which received no drugs or vehicles; sham group (n=10), which received streptozotocin solution; and diabetic group (n=15), which received streptozotocin. Eight weeks after diabetes mellitus induction, the animals were weighed and anesthesized; blood samples were collected from abdominal aorta for plasma total homocysteine, von Willebrand factor and glucose levels.

Results: The experimental model was reproducible in 100% of animals. The mean plasma homocysteine levels were: 7.9 μmol/l (control), 8.6μmol/l (sham) and 6.1μmol/l (diabetic), with difference among the groups (p<0.01). Multiple comparison analysis among the groups showed that values in the diabetic group were lower than in the sham group (p≤0.01). The mean von Willebrand factor values were 0.15 U/l (control), 0.16U/l (sham) and 0.18 U/l (diabetic), with difference among the groups (p=0.03). The mean value was higher in the diabetic group than in the control group (p≤0.05). Correlation between homocysteine and von Willebrand factor was not observed in the diabetic group.

Conclusion: Reduced homocysteine levels and increased von Willebrand factor levels were observed in diabetes mellitus induced by streptozotocin; nevertheless, there were no correlations between them and with final glucose levels.

Key words: Homocysteine/blood; von Willebrand factor; diabetes mellitus; epidemiology; experimental.

Introduction

Diabetes mellitus (DM), a disease characterized fundamentally by a disorder in the metabolism of carbohydrates, is usually accompanied by macroangiopathy and microangiopathy, peripheral, cranial nerve and autosomal neuropathy, which cause isolated or associated major clinical complications with a high mortality rate.

In terms of vasculopathy, the endothelium is primarily affected and several elements have been related to its dysfunction.

The purpose of this article is to study the possible changes in plasma levels of homocysteine (He) and von Willebrand factor (vWF), which are known to be related with the pathogenesis of endothelial dysfunction, in rats with streptozotocin-induced (STZ) diabetes mellitus.

Methods

This is an experimental study involving adult male rats (*Rattus norvegicus albinus, Rodentia, mammalia*) at same age range and weight between 180-200g. The animals were weighed at the beginning of the experiment and then on a weekly basis up to the day they were killed. They were randomized into 3 groups: Group I (control): the animals continued to receive a normal diet and were allowed free water intake; Group II (sham): the animals received only the saline solution of STZ (citrate buffer 0.1M, pH = 4.5) and remained with the same local and food condition as the animals in Group I; Group III (diabetes): received the injection of STZ (Sigma® IP).

DM was induced with intraperitoneal administration of STZ (single dose of 60 mg/kg body weight) diluted in 0.3 mL of 0.1 M citrate buffer solution[1-3]. During the first 48 hours, diabetic rats were offered glucose solutions (2.5% and 5%) which replaced water in order to prevent hypoglycemia due to hyperinsulinemia resulting from the destruction of pancreatic beta cells. During the first 72 hours after administration of STZ, blood glucose was measured every day at the same time to prove the method reproducibility; measurements were made with Advantage (Roche®) meter by using blood collected from a vein puncture in the animal tail. Rats with glucose levels ≥250 mg/dL were considered diabetic[1-5].

Eight weeks after DM induction, the animals were weighed, their blood glucose levels were checked and the animals underwent general anesthesia with intraperitoneal administration of thionembutal (Sigma®) (50 mg/kg body weight) for further blood collection and killing.

Correspondência: Renato Delascio Lopes •
Rua Pedro de Toledo, 980, 11º andar, conj. 116 – Vila Clementino
04039-002 – São Paulo, SP - Brazil
E-mail: rdlopes@climed.epm.br
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Blood collection was performed by means of puncture of the abdominal aorta and 3 mL of blood were distributed into tubes containing EDTA as an anticoagulant agent, followed by centrifugation at 3000 rpm. Plasma was aliquotted into 1-mL vials and stored in a freezer at –20ºC until determination of plasma total He levels. The remaining blood was placed in a dry tube, centrifuged and the serum was aliquotted into 1.5-mL vials, and frozen at –20 °C for determination of vWF and glucose levels.

Determination of plasma total He was carried out with high performance liquid chromatography (HPLC) by fluorimetric detection, following a standardized protocol based on the methodology described by Pfeiffer et al[6] and used by Cruz et al[7]. Plasma underwent 3 stages of preparation: reduction, protein precipitation and derivatization. We used the system from Shimadzu®, composed of an autoinjector model SIL-10 Advp, pumps and fluorescence detector model RF – 10AXL. Separation was performed with a Prodigy ODS2 column, measuring 150 x 3.2 mm and containing 5μm microparticles (Phenomenex®) and a pre-column of Adsorbosphere® C18 measuring 7.5 x 4.6 mm and containing 5μm microparticles (Alltech®). Dectection was performed with the detector adjusted for excitation at 385 μm and emission at 515 μm.

Measurement of the initial fasting blood glucose (initial BG) and at the end of the experiment (final BG) we used the enzymatic-colorimetric method, glucose oxidase/peroxidase (GOD PAP-Celm). The reference values were considered in the range of 75-99 mg/dL. The von Willebrand factor was determined by Elisa technique using a von Willebrand factor kit (DAKO®).

The study was evaluated and duly approved by Research Ethics Committee under record No.1341/05. In the statistical analysis, in order to assess if the mean variables studied were the same in the three groups, we used an analysis of variance (ANOVA) with a fixed factor (group) and three levels (control, sham and diabetes). For the final blood glucose variable, we used reverse transformation to stabilize variance. When ANOVA showed that the means in the groups were not identical, we used Tukey's procedure for multiple comparisons to identify which groups were responsible for such differences. Dispersion diagrams were built and the Pearson correlation coefficients were calculated to assess the presence of linear association between the variables. The significance level was established at 5% in all analyses.

Results

The DM experimental model was reproducible in 100% of animals. Table 1 shows the mean, standard deviation, median, minimum and maximum values for the variables in each group. Figures 1-4 contain box-plots for such variables.

Table 1 also presents the descriptive level of analysis of variance employed to compare the means in the three

Table 1 – Descriptive measurements for initial and final blood glucose (BG), homocysteine (Hc) and von Willebrand factor (vWf) in each group, and the ANOVA descriptive level

		Control	Sham	Diabetes	Descriptive level
He	Mean	7.9	8.6	6.1	<0.01
	Standard deviation	2.3	2.2	1.3	
	Minimum	4.2	6.4	3.2	
	Median	7.7	7.8	6.3	
	Maximum	12.0	13.0	8.1	
vWF	Mean	0.15	0.16	0.18	0.03
	Standard deviation	0.3	0.2	0.4	
	Minimum	0.11	0.13	0.14	
	Median	0.15	0.15	0.18	
	Maximum	0.19	0.19	0.26	
Initial BG	Mean	88.7	88.9	85.1	0.23
	Standard deviation	5.9	8.2	5.2	
	Minimum	80.0	80.0	80.0	
	Median	90.0	88.0	83.0	
	Maximum	99.0	101.0	98.0	
Final BG	Media	85.0	80.9	353.5	<0.01
	Standard deviation	7.1	5.0	98.2	
	Minimum	76.0	75.0	245.0	
	Median	84.5	79.5	349.0	
	Maximum	99.0	91.0	561.0	

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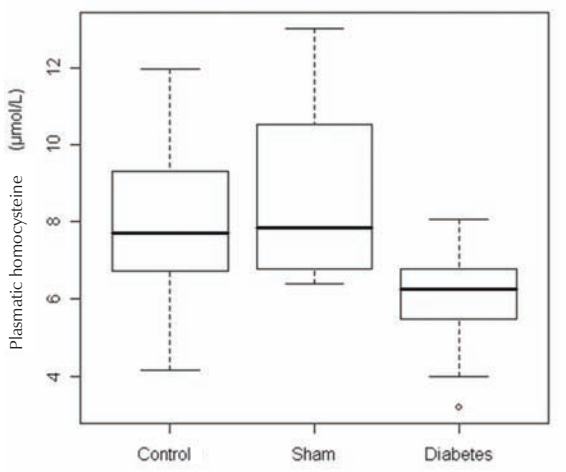

Fig. 1 - *Plasma total homocysteine in each group (box-plot). Differences found (p<0.05): Diabetes vs Control; Diabetes vs sham.*

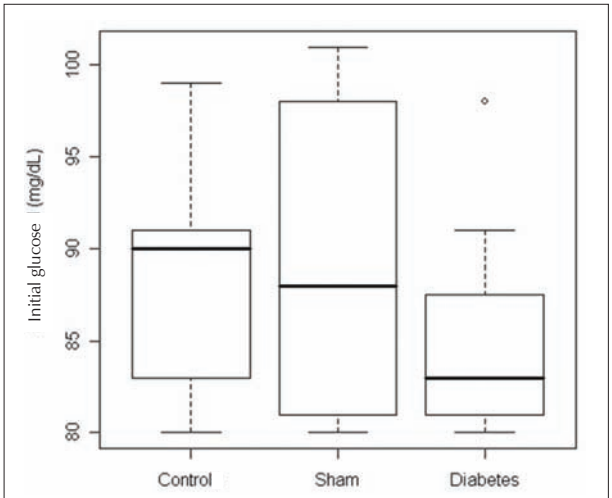

Fig. 3 - *Initial fasting glucose level in each group (box-plot). No difference among the means was detected (p=0.23).*

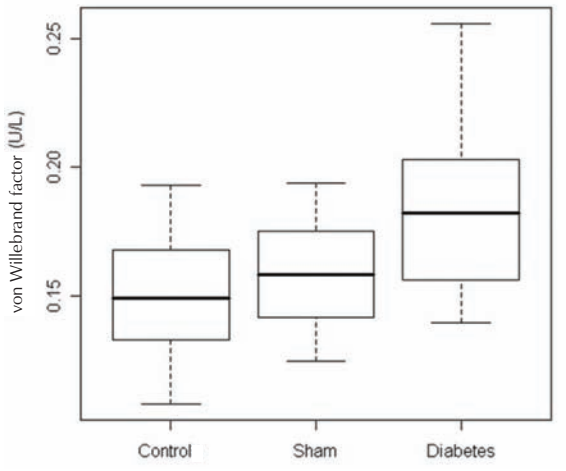

Fig. 2 - *von Willebrand factor in each group (box-plot). Differences found (p<0.05): Diabetes vs Control.*

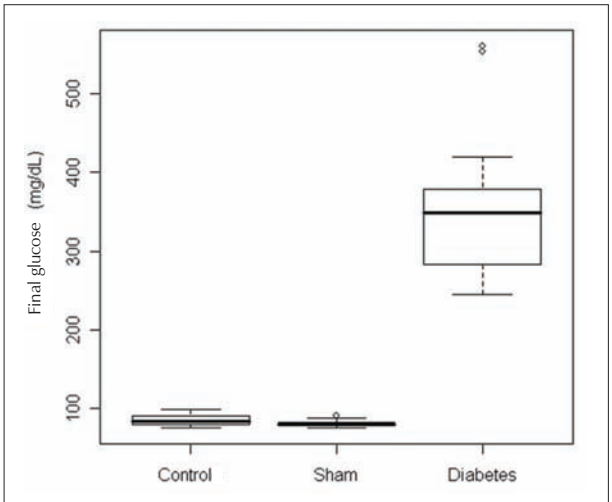

Fig. 4 - *Final fasting glucose level in each group (box-plot). Differences found (p<0.05): Diabetes vs Control; Diabetes vs sham.*

groups, for each variable. According to this analysis, there was no difference among the means of initial blood glucose levels in the three groups (p= 0.23). Regarding other variables (plasma He, vWF and serum concentration of blood glucose on the day of killing), the means were not the same in the three groups (p<0.01, p= 0.03 and p<0.01, respectively).

According to Tukey's procedure for multiple comparisons, the mean He was lower in the group of diabetic animals than in the control and sham groups; there was no difference between the means of control and sham groups (p<0.05). As to vWF, the mean was higher in the diabetes group than in the control group; no other difference was detected among the groups (p<0.05). For final glucose level, the mean was higher in the diabetes group than in the control and sham groups, and there was no difference between the means of control and sham groups (p<0.05).

Table 2 presents the Pearson correlation coefficients among the variables studied for the diabetes group. No correlation was observed among the variables plasma He, vWF and blood glucose at the end of the experiment.

Table 2 - Pearson correlation coefficients among the variables studied in each group

Diabetes	He	vWF[1]
vWF	0.13*	
Final glucose	-0.06*	0.18*

** NS; vWF - von Willebrand factor.*

Discussion

DM is a disease responsible for numerous cardiac and vascular complications. Endothelial dysfunction is an important element in its pathophysiology and several biochemical abnormalities are involved in this condition.

Homocysteine (He) was first reported as a cause of atherosclerosis in 1969[8], and has been gaining more importance as a risk factor for coronary artery disease[9-15] with its intriguing possible participation in the pathogenesis of endothelial dysfunction. An amino acid formed exclusively by demethylation of dietary methionine or by catabolism, it contains a thiole group (SH-) and does not participate in protein synthesis[16]. Only 2-5% of free plasma He is found in its reduced form and 70-80% is found in the bloodstream bound to plasma proteins, especially albumin.

The prevalence of hyperhomocysteinemia (HHe) is 5-7% in the overall population and intermediate levels are found in 13-47% of individuals with symptomatic atherosclerotic vascular disease[17-19]. Several publications correlate HHe with coronary, cerebral and peripheral artery disease, as well as venous thrombosis, and it has shown to be an important cardiovascular risk factor, independent from the others[20].

A meta-analysis involving 27 studies with more than 4000 patients showed that, for He values higher than 10 μmol/L, an increase by 5 μmol/L of circulating He is associated with a higher risk of cardiovascular disease. A He increase of 5 μmmol/L corresponds to an increase by 20 mg/dL in plasma total cholesterol, with a higher likelihood of acute myocardial infarction[21]. Graham et al[22] concluded that fasting HHe or after methionine overload corresponds to a cardiovascular risk that is similar to that of hyperlipidemia or smoking, although lower than that of systemic hypertension.

In 1998, Folsom et al[23] questioned the participation of HHe in the pathophysiology of coronary artery disease in an important study. However, their results are not corroborated by more recent studies[24].

The pathogenesis of the vascular injury caused by HHe includes damage to the endothelial cell, growth of vascular smooth muscle, higher platelet adhesion, increased oxidation of LDL-cholesterol with deposits in the vessel wall and direct activation of the coagulation cascade[20]. However, it is still not clear by which pathophysiological mechanism He promotes the development of atherothrombosis[25].

The endothelium, the main endocrine organ in the body, is of great importance in numerous degenerative and inflammatory diseases. Among several methods to appraise its dysfunction, vWF has been very efficient and presents excellent sensitivity. Endothelial cells and megacariocytes synthesize, store and secrete vWF. Such secretion increases when the endothelial cells are stimulated or damaged, and the values can increase by two- to ten-fold in affected individuals. The von Willebrand factor binds to collagen and to other components of the vascular wall and, therefore, it serves as a mediator of platelet adhesion to the subendothelium of damaged vessels[26].

Vascular injury exposes the subendothelial collagen matrix, which binds to the circulating vWF in the plasma; this, in turn, binds to the extracellular platelet membrane glycoproteins (GPIb), whose intracellular portion is bound to the filamin of the platelet cytoskeleton. This interaction, known as platelet adhesion, is enough to trigger two phenomena in the platelets: cell contraction and conformational abnormality[27-29].

In DM, because of genetic and plasma biochemical factors, endothelial dysfunction is early and determines the beginning and progression of vascular disease, thus causing diabetic microangiopathy and macroangiopathy. In a recent study, Becker et al[25] aimed to determine to which extent He was associated with endothelial dysfunction in diabetic and non diabetic rats, and whether such a dysfunction could be estimated by plasma vWF levels. They concluded that the relation between He and atherothrombosis can not be explained by the association of homocysteine and vWF. In the search for an element that can be a marker of this damage, vWF starts to have an important clinical interest and it can be considered a serum marker of endothelial dysfunction in DM.

For induction of DM, we used STZ in this experiment for being the model that represents better reproducibility of results as compared with those found in the literature[30-35]. The results of this study show that there are changes in the plasma He and vWF levels in DM, due to metabolic abnormalities, among which hyperglycemia is the most important.

Similar to the results of the current study (Table 1), the studies performed by Jacobs et al[36], Wollesen et al[37] and Veldman et al[38] demonstrated lower levels of He in rats with experimental diabetes.

Since homocysteine causes direct damage to the endothelium, it also stimulates cell proliferation and collagen production, maintains a chronic vascular inflammatory process, changes the endothelium-dependent arterial relaxation by means of nitric oxide, increases oxidative stress, activates coagulation factors and inhibits protein C activity. Therefore, we would expect to find increased levels of He in diabetes due to the extensive endothelial damage. However, in the presence of hyperglycemia, there is an increase in glomerular filtration[37-38]; the metabolic changes in He pathway[36] result in lower levels, which corroborates the assertion that He is a risk factor for endothelial dysfunction and not a specific marker of endothelial damage in DM. We can infer that normal or increased values of He in DM are related with endothelial injury.

The von Willebrand factor has been optimized as a marker of endothelial dysfunction. In the current study, its values (Table 2) were significantly increased in diabetic rats, which make us consider it is an important factor in DM endothelial dysfunction.

No correlation between He and vWF (Table 2) has been observed; therefore, we conclude that in this experiment the relationship between He and possible atherothrombosis in diabetic rats can not be explained by the association between He and vWF.

Conclusions

Hemocysteine values were lower in diabetic rats, and could trigger or maintain endothelial damage; therefore, it is not a marker of its dysfunction. Since vWF is a marker of endothelial

dysfunction, its increased levels allow inferring the presence of vascular damage in DM. There was no correlation between final blood glucose, He and vWF in diabetic rats.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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