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The Effects of Folic Acid Administration on Cardiac Tissue Matrix Metalloproteinases Activities and Hepatorenal Biomarkers in Diabetic Rats

Slavica Mutavdzin¹, Kristina Gopcevic², Sanja Stankovic³, Jovana Jakovljevic Uzelac¹, Milica Labudovic Borovic⁴ and Dragan Djuric¹

¹Institute of Medical Physiology "Richard Burian", Faculty of Medicine, University of Belgrade, Belgrade, Serbia

²Institute of Chemistry in Medicine "Prof. Dr. Petar Matavulj", Faculty of Medicine, University of Belgrade, Belgrade, Serbia

³Centre of Medical Biochemistry, Clinical Centre of Serbia, Belgrade, Serbia

⁴Institute of Histology and Embryology "Aleksandar Dj. Kostic", Faculty of Medicine, University of Belgrade, Belgrade, Serbia

Correspondence to:

Dragan M. Djuric, MD, PhD

Institute of Medical Physiology "Richard Burian",

Faculty of Medicine, University of Belgrade,

Visegradska 26/2, 11000 Belgrade, Serbia

Phone number: +381113607112

Fax number: +381113611261

e-mail: drdjuric@eunet.rs, dr_djuric@yahoo.com

Abstract

The aims of this study were to investigate the influence of diabetes mellitus (DM) and folic acid treatment on the liver and renal biomarkers, and on heart remodeling through evaluation of cardiac matrix metalloproteinase (MMPs) activities.

There were four groups: C – control (physiological saline 1ml/kg, i.p. 28 days), DM – diabetes mellitus (STZ 100mg/kg in physiological saline, i.p. one day), FA – folic acid (5mg/kg in physiological saline, i.p. 28 days), and DM+FA – DM and folic acid group (STZ 100mg/kg in physiological saline, i.p. one day and folic acid 5mg/kg in physiological saline, i.p. 28 days). Our results demonstrated increased aminotransferases, and alkaline phosphatase activities, urea and creatinine and decreased albumin and fibrinogen concentration in the DM group. MMP-2 relative activity was elevated in the DM and FA group, while MMP-9 was decreased in the DM and increased in the FA group. The folic acid treatment of diabetic rats did not change aminotransferases activities; it alleviated the increase in alkaline phosphatase and the decrease in albumin and fibrinogen concentration, and significantly reduced MMP-2 activity; however, it increased urea and creatinine concentrations.

In conclusion, folic acid treatment of diabetic rats has cardio- and hepatoprotective effects. Nevertheless, folic acid dosing should be carefully considered because of possible renal damage.

Key words: Diabetes mellitus, cardiac remodelling, matrix metalloproteinases, hepatic damage, renal damage, rat

Introduction

Diabetes mellitus (DM) is a global public health problem with an increasing incidence and prevalence (Danaei et al. 2011). It is a group of metabolic disorders characterized by hyperglycemia, altered insulin action or secretion, changed metabolism of proteins, carbohydrates, and lipids, as well as by the increased risk of vascular complications (Chaiyasut et al. 2011). Because of possible DM complications it can lead to severe and irreversible pathological conditions such as vasculopathy, hepatopathy, nephropathy, retinopathy, neuropathy, and cardiovascular diseases (Reid 2006).

It is known that endothelial dysfunction occurs in patients with DM and that it plays an important role in the development of atherosclerosis (Umadevi et al. 2016). In endothelium damage and dysfunction an increase in the plasma levels of endothelial markers, such as von Willebrand factor (vWF) occurs (Umadevi et al. 2016). One of the long-term complications of DM and vasculopathy is diabetic nephropathy (Pop-Busui et al. 2010) and hepatopathy (Reid 2006). DM is also followed by cardiovascular complications and cardiac remodeling that can lead to heart failure. Pathogenetic mechanisms of diabetic cardiomyopathy are different, but usually they include metabolic disturbances, insulin resistance, microvascular disease, renin-angiotensin system activation, and oxidative stress (Min et al. 2018; Pappachan et al. 2013). Its main morphological characteristics are cardiac hypertrophy and remodeling which are related with myocardial fibrosis (Min et al. 2018).

It is considered that matrix metalloproteinases (MMP) have an important role in degradation of extracellular proteins and remodeling in the myocardium (Ichihara et al. 2006). The MMPs are part of proteolytic enzymes family that consists of more than 20 members. They can be divided in the interstitial collagenases (MMP-1 and -13), the gelatinases (MMP-2 and -9), and membrane-associated enzymes (membrane type 1-MMP) (Visse and Nagase 2003). Earlier studies in animal models of heart failure have demonstrated significant activation of MMP-2

(Bergman et al. 2007). There is a small number of papers with controversial results describing the activities of MMPs in the cardiac tissue of diabetic rats or mice. Some authors have demonstrated increased activities of both MMP-2 and MMP-9 (Min et al. 2018; Rodriguez et al. 2006; Bhatt and Veeranjanyulu 2012), while others have shown decreased MMP-2 and increased MMP-9 (Silva et al. 2017) activities or protein concentrations (Westermann et al. 2007). Changes in activities and expression of MMPs can be connected with increased oxidative stress in diabetes (Akat et al. 2018). Folic acid administration reduces oxidative stress, improves endothelial function, and prevents apoptosis by reducing plasma homocysteine level (Hou et al. 2018; Wald et al. 2006). It is also demonstrated a decrease in apoptosis of cardiomyocytes after the administration of the folic acid in diabetic rats (Wu et al. 2008).

The aims of this study were to investigate the DM influence on the liver and renal biomarkers in serum or plasma, and on heart remodeling through evaluation of cardiac MMPs activities, as well as to evaluate the effects of folic acid administration in healthy and diabetic rats on these parameters.

Materials and Methods

Experimental Animals

Male Wistar albino rats (n = 38) with a body mass of approximately 160 g at the start of experimental period were used in the research. All experimental animals were housed at constant ambient temperature $21 \pm 2^{\circ}\text{C}$ with $55 \pm 5\%$ relative humidity in a 12/12-hour light-dark cycle with the light period beginning at 07:30 a.m. They were housed in pairs in transparent plexiglas cages with a wood-chip floor and standard rat chow and water were provided *ad libitum* throughout the study period. The study has been approved by the Ethical Council for the Welfare of Experimental Animals, Ministry of Agriculture, Forestry and

Water Management, Veterinary Directorate, Republic of Serbia (Number: 323-07-01339/2017-05/04). All experimental procedures were done in accordance with the prescribed legislation (EU Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes 86/609/EES) and the principles of ethics.

Experimental Protocol and DM induction

The research was conducted during a four-week period. The animals were randomly allocated into one of the four groups. The first group was the control group (C, $n = 10$) that received physiological saline treatment during 28 consecutive days (1 ml/kg b.w., i.p.). The second group was the group of experimental animals with induced *diabetes mellitus* (DM, $n = 8$) using one dose of streptozotocin (STZ, 100mg/kg b.w. in physiological saline, i.p.). The third, folic acid group (FA, $n = 10$), received folic acid (5mg/kg b.w. in physiological saline, i.p) treatment during 28 consecutive days. The fourth group was the group of experimental animals with induced DM and folic acid treatment (DM + FA, $n = 10$). First they were treated with STZ (100mg/kg b.w. in physiological saline, i.p., in one dose) and on the fourth day after that the folic acid treatment started and lasted during the next 28 consecutive days. Folic acid treated animals have received folic acid in the dose of 5 mg/kg during 28 days (Mohammadian et al. 2015). The diet containing folic acid 2 mg/kg is generally accepted as the basal dietary requirement for rats (National Research Council (US) Subcommittee on Laboratory Animal Nutrition 1995). If the diet contains 8 mg/kg folic acid, it provides moderate folic acid supplementation, and it is selected to approximate 1-6 mg/day of folic acid in humans. These doses correspond to the recommended doses that may be consumed in humans (Partearroyo et al. 2015). According to these recommendations and our previous experience we used 5 mg/kg/day dosage of the folic acid.

Diabetes mellitus was induced in experimental animals by an intraperitoneal injection of freshly prepared STZ (100 mg/kg b.w.), dissolved in physiological saline (0.9 % NaCl, 1

ml/kg b.w.). Citrate buffer is used for dissolving STZ more frequently than physiological saline (Gimenes et al. 2018; Gimenes et al. 2015). In order to avoid the effects of citrate buffer on the tested parameters, we used a physiological saline to dissolve STZ, but because of that we applied a higher dose of STZ. After 72h fasting blood glucose level was measured, and a glucose level higher than 12.2 mmol/l was considered as a positive for DM (Gimenes et al. 2018). In order to confirm the development of diabetes another control group (C0, n = 8) that received physiological solution in one dose and the DM group were used. In these groups serum glucose and insulin level were measured four weeks after hyperglycemia development. The serum insulin level was determined by the RIA method, using rat insulin standards. The rat fasting insulin reference level was 12.06–48.26 mU/l (Tepavčević et al. 2014). Also, insulin resistance was evaluated by calculation of homeostasis model assessment of insulin resistance (HOMA-IR) (Matthews et al, 1985).

The body mass and blood glucose level were measured at the start, weekly and at the end of the experimental period. After four weeks of the treatment euthanasia was performed using a rat guillotine and rat blood samples were collected by exsanguination. Blood was collected through a glass funnel and placed in appropriate vacutainers with anticoagulant (sodium citrate) to isolate plasma or without anticoagulants to isolate serum samples. The blood samples were left at the room temperature for 15 min and afterwards centrifuged (15 min×3000 rpm). The obtained plasma and serum were used for the analyses. Prior to blood samples collection, animals were fasted overnight to avoid food induced changes in the blood biochemical parameters. Whole hearts were isolated from all the rats. The hearts were rinsed in the physiological saline and weighed. After this procedure the hearts were homogenized and centrifuged for further analyses.

Biochemical Parameters in Serum or Plasma Determination

The glucose level in blood obtained from the rat tail vein was determined by an ACCU-CHEK analyzer (Roche Diagnostics, Indianapolis, USA). Serum homocysteine was measured by an automated electrochemiluminescence immunoassay system ADVIA Centaur XP system (Siemens Healthcare GmbH, Erlangen, Germany). The serum glucose level, liver enzymes activities (aspartate aminotransferase – AST, alanine aminotransferase – ALT, and alkaline phosphatase – ALP), total proteins, albumin, alpha-amylase (α -amylase) and renal parameters (urea and creatinine) were analyzed using spectrophotometry and commercial kits (Siemens Healthcare Diagnostics Ltd. Frimley, Camberley, UK) on an automatic biochemical analyzer (Dimension Xpand, Siemens). For determination of haemostatic factors (fibrinogen concentration and activity of vonWillebrand Factor – vWF) immunoturbidimetric commercial assay (Siemens Healthcare GmbH, Marburg, Germany) was used.

Preparation of the Cardiac Tissue Samples

Cardiac tissue (100 mg) was homogenized on ice in 1 ml of buffer (20 mmol/l Tris-HCl, pH 7.5, 250 mmol/l sucrose, 1 % Triton X-100, with addition of protease inhibitor: 1 mmol/l phenylmethylsulfonyl fluoride (PMSF) and 1 μ g/ml leupeptin) (Rouet-Benzineb al. 1999). The homogenate was centrifuged at 10000 rpm at 4 °C during 10 min. In this way the proteins from the cardiac tissue were isolated. The total protein concentration was measured using the Bradford method (Bradford 1976).

Cardiac tissue MMP-2 and MMP-9 Relative Activities Evaluation

MMP relative activities were evaluated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Cardiac tissue samples were diluted 10 times in 200 g/l sucrose to prepare the test samples. The samples were analyzed by SDS-PAGE zymography according to the method of Kleiner and Stetler-Stevenson (1994). For each sample, the same amount of total tissue protein (6.25 μ g) was loaded. Test samples were incubated for 40 min at 37 °C and electrophoresis was performed without reduction on 7.5 % polyacrylamide gels

copolymerized with 0.1 % gelatine at 4 °C at a constant current of 50 mA. When the tracking dye at the front reached the bottom of the gel, the gel was removed and shaken gently for 45 min in 0.25 g/l Triton X-100 to remove SDS. Then, the gel slabs were transferred to a bath (without Triton X-100) and washed twice for 20 min to remove Triton X-100. Then, the gels were incubated and shaken for 24 h in 0.1 mol/l glycine, 50 mmol/l Tris-HCl, 5 mmol/l CaCl₂, 1 mol/l ZnCl₂, 0.5 mol/l NaCl, at pH 8.3, at 37 °C. Afterwards gels were stained with Coomassie brilliant blue G-250 dye during 3 h, regions of proteolytic activity were visualized as clear zones against a blue background (Laemmli 1970). Following gelatin zymography, gels containing samples were scanned for densitometric analysis in order to quantify the relative activity of the two gelatinases (MMP-2 and-9). The degree of gelatin digestion was quantified as enzymes activities using ImageJ Q.42 software package. Densitometric data were then normalized for 1 µg of total cardiac tissue proteins.

Statistical Analysis

Data were expressed as mean ± SEM or median (minimal value; maximal value). Statistical comparison between the values obtained from experimental groups was performed by one-way analysis of variance (ANOVA), followed by Tukey's Post Hoc Test or by the Kruskal-Wallis test and Mann-Whitney U depending on the data distribution. The Pearson correlation coefficient was also calculated in order to determine the correlation between body and heart mass. SPSS 19.0 for Windows software package was used for statistical analyses. Differences were considered significant at $P < 0.05$.

Results

DM Development Confirmation

Serum glucose and insulin level, as well as HOMA-IR were determined in the C0 and the DM group to confirm the development of diabetes. In the DM group insulin level was significantly

lower and glucose level and HOMA-IR were significantly higher ($P < 0.001$) than in the C0 group (Table 1).

Body and Heart Mass

There were no lethal cases over the entire experimental period. Body mass did not differ between the groups at the beginning of experiments, but significant weight loss was observed in both diabetic rats groups during experimental period ($P < 0.001$, Fig. 1). Also heart mass was significantly lower ($P < 0.001$) in the DM (640.9 ± 31.3 mg) and DM + FA group (660.3 ± 30.5 mg) in comparison to the C (1100.2 ± 37.0 mg) and FA group (1126.7 ± 50.3 mg). Statistically strong linear correlation of heart and body mass was observed in the C and in both diabetic groups (C: $P = 0.036$, $r^2 = 0.666$; DM: $P < 0.001$, $r^2 = 0.955$; DM + FA: $P = 0.031$, $r^2 = 0.678$), but not in the FA group (FA: $P = 0.6$, $r^2 = -0.19$) (Fig. 2).

Biochemical Parameters in Serum or Plasma

The glucose level was without statistical difference between the tested groups before the STZ treatment ($P = 0.138$). It was elevated in all STZ treated animals 72 h after the administration (DM = 17.2 ± 0.65 mmol/l and DM + FA = 18.8 ± 1.35 mmol/l). At the end of experimental period glucose level in the group DM + FA was still elevated in comparison to the healthy rats, but significantly lower than in the DM group (DM = 27.7 ± 2.18 mmol/l and DM + FA = 19.2 ± 3.12 mmol/l, $P = 0.022$), (Fig. 3). Statistically significant difference in serum homocysteine level was found among the groups ($P < 0.001$, Table 2). A Fig. 4. shows serum liver enzymes activities. The activities of serum ALT, AST, and ALP did not change in non-diabetic rats; however, their values were elevated in both diabetic groups. There were no differences in AST and ALT activities between the DM and the DM + FA group, while folic acid treatment induced significant decrease in ALP activity.

Serum total protein level did not change in the groups, while serum albumin concentration was significantly lower in the DM and DM + FA group comparing to the C and FA group (P

< 0.001, Fig. 5). Albumin concentration was significantly increased in the DM + FA group in the comparison to the DM group ($P = 0.022$). Alpha-amylase activity was significantly decreased in both diabetic groups (Table 2, $P < 0.001$). Fibrinogen was significantly decreased in the DM group (1.95-fold in comparison with the C group), while its value did not change in other groups (Table 2). Another haemostatic parameter, vWF was increased in both diabetic groups (Table 2, $P < 0.001$). Serum renal parameters, urea and creatinine, show differences between tested groups (for all three parameters $P < 0.001$, Fig. 6). In the comparison to the C and FA group urea concentration was elevated in the DM (2.3 -folds), and especially in the DM + FA group (almost 3-folds), while creatinine concentration was increased only in the DM + FA group (2-folds).

Cardiac tissue MMP-2 and MMP-9 Relative Activities Evaluation

Representative zymographs of cardiac tissue MMP-2 and MMP-9 activities detected by SDS-PAGE zymography are demonstrated in Fig. 7A. Latent form of MMP-2 (proMMP-2 at 72 kDa) can be detected in all tested groups, while active form of MMP-2 (aMMP-2 at 64 kDa) was detected only in the DM group. ProMMP-2 relative activity was 66 % and 58 % higher in the DM and FA group, respectively, than in the C group, while folic acid treatment reduced this activity in diabetic rats to the values that are 10 % higher than the activity in the C group (Fig. 7B). Active form of MMP-9 (aMMP-9 at 83 kDa) was detected in all tested groups, but with significant difference in activities between the groups. In the DM group aMMP-9 activity was 11 % lower than in the C, whereas in the FA and DM + FA group this activity was higher 72 % and 9 %, respectively than in the C group (Fig.7B).

Discussion

Experimental DM can be induced in different ways, such as chemically, spontaneous autoimmune induced, genetically, and virally (King 2012). Chemically induced diabetes is a

simple and relatively cheap model of diabetes development in rodents. In these models of DM type I, a high percentage of the endogenous beta cells are destroyed, and consequently, there is little endogenous insulin production, leading to hyperglycaemia. STZ and alloxan are the most frequently used compounds to chemically induce DM (King 2012). Both chemicals are relatively unstable, and the solutions should be made immediately prior to i.p. or i.v. injection. STZ is synthesized by *Streptomyces achromogenes*. After administration, it enters the pancreatic beta cells through the Glut-2 transporters and provokes alkylation of the DNA, reduction in ATP and inhibition of insulin production (Szkudelski 2001, Sandler and Swenne 1983). STZ is a source of free radicals that can also contribute to DNA damage and subsequent cell death. Chemically induced DM is appropriate to use when testing drugs or therapies where the main mechanism of action is lowering blood glucose in a non-beta-cell-dependent manner. DM is usually induced several days before the start of the experiment to ensure stable hyperglycaemia (King 2012).

In our study increased glucose and HOMA-IR and decreased insulin level confirmed the development of diabetes in the DM group. All the animals treated with STZ have elevated glucose level 72 h after the STZ administration. Application of folic acid has positive effect on glucose level since it attenuates hyperglycaemia (however, glucose level values remained still higher than 11 mmol/l) in diabetic rats. DM type I is characterized by body mass loss (Gimenes et al. 2015; Savi et al. 2017). All our diabetic animals had decreased body mass, and the folic acid treatment did not have any influence on it. Also heart masses were decreased in diabetic animals. Interestingly, statistically strong linear correlation of heart and body mass was observed in the C and in both diabetic groups but not in the FA group. Earlier studies have demonstrated that there is decreased heart mass expressed per 100 g of body in rats fed with a low protein diet (Debski et al. 2007) and in the heart masses of mice fed with a

high-fat diet (Li et al. 2017) that were treated with folic acid. This indicates that folic acid may have influence on the heart mass and correlation between the heart and body mass.

Measuring biochemical serum parameters, the results reveal that homocysteine level was elevated in the FA group and decreased in the DM + FA group. Increased homocysteine level in the healthy rats after the folic acid treatment was not expected and mechanism that induced these results remains unclear. Other authors have demonstrated that folic acid has positive effects by reducing homocysteine level. These effects reduce oxidative stress, improve endothelial function, and prevent apoptosis (Hou et al. 2018; Wald et al. 2006). Hou et al. (2018) have obtained lower homocysteine levels in folic acid treated diabetic rats; also in patients with DM and hyperhomocysteinemia the folic acid treatment lowered plasma homocysteine level and improved endothelial function (Hayden and Tyagi 2004; Title et al. 2006). Their results are in accordance with ours, which demonstrate lower homocysteine concentration in the DM + FA group than in the DM group.

Since DM is defined as a metabolic disorder, changes in the function of liver, the main metabolic organ, are expected in patients with this disease. Serum ALP, AST and ALT levels represent parameters that can be easily determined in order to evaluate the hepatic function (Degirmenchi et al. 2002). Serum activities of all three tested enzymes were evaluated in diabetic rats. Folic acid treatment induced significant decrease in ALP activity, while there was no influence of folic acid on AST and ALT activities. Increases in the serum activities of these enzymes are sign of active liver damage (de Gouveia et al. 2017) and a common sign of different liver diseases (Arkkila et al. 2001). ALP will be released in circulation and its level will be increased after cellular damage since it is located in cytoplasm (Sallie et al. 1991). Aminotransferases are released in circulation when there is cellular injury that involves also the injury of mitochondria (Senthil et al. 2003). It is demonstrated that increased aminotransferase activities are more frequent among people with diabetes than in the general

population (Arkkila et al. 2001). Other authors have also obtained significantly increased serum levels of AST, ALT and ALP in diabetic rats that may be the consequence of the hepatocellular damage caused by STZ-induced diabetes in experimental cases (Zafar et al. 2009; Saeed et al. 2008; Latifi et al. 2019). Chayarop et al. (2017) demonstrated significantly increased serum ALP in diabetic rats indicating liver damage, while transaminases did not differ from healthy controls.

This study demonstrates that total protein level does not differ between the groups, while serum albumin concentration is significantly lower in the DM and DM + FA group in comparison to the C and FA group. Decreased albumin concentration that is synthesized by hepatocytes may occur as a consequence of liver damage, increased protein catabolism or increased protein loss through urine (Chayarop et al. 2017). Other authors obtained also decreased serum albumin concentration (Xue et al. 2017; Chayarop et al. 2017), while Chayarop et al. (2017) demonstrated that in addition to low albumin, there is a reduction in serum total protein. In another study there were no differences in both total protein and albumin value between diabetic and control rats (Kuga et al. 2018). Our study reveals significant increase in serum albumin in diabetic rats after the folic acid treatment. Fibrinogen, which is a liver product and haemostatic factor, was significantly decreased in the DM group, while folic acid prevented its decrease in diabetic rats. These results are in agreement with the serum ALP level decrease by the folic acid administration. This indicates the protective effects of folic acid against liver damage that are associated with hyperglycaemia (Chayarop et al. 2017). Another haemostatic parameter, vWF was increased in the DM as well as in the DM + FA group without significant difference between these two groups. vWF is endothelial marker that plays an important role in hemostasis under physiologic conditions. However, under pathologic conditions, vWF plasma level may be used as a biomarker of endothelial damage and dysfunction, since it is secreted almost only by

endothelial cells (Frankel et al. 2008; Meigs 2010). Previous studies demonstrated increased vWF level in type 2 diabetic patients or in insulin resistance (Frankel et al. 2008; Umadevi et al. 2016), as well as associated increased risk of cardiovascular diseases with higher levels of vWF in these populations (Frankel et al. 2008).

Alpha-amylase activity was significantly decreased in both diabetic groups, but especially in the DM + FA. Its activity was also decreased in the FA group. It was found that folic acid binds alpha-amylase with non-covalent bonds and they form complexes. After molecular modeling it was demonstrated that folic acid binds into the primary substrate-binding pocket and occupies the active site of α -amylase leading to the enzyme inhibition. The formation of these complexes results in reducing the enzymatic activity of α -amylase (Shi et al. 2017). Our results indicate that the decrease in α -amylase activity may be the consequence of possible pancreatic damage and folic acid treatment, since this enzyme activity was the lowest in the DM + FA group.

One of the most common and most serious microvascular complications of DM is diabetic nephropathy (Sun et al. 2018) and it is the leading cause of end-stage renal disease (Pop-Busui et al. 2010). In animal models of diabetic nephropathy, dysfunction of nitric oxide synthase (NOS) is a main contributor to increased oxidant stress (Sato et al. 2005). *In vitro* studies have demonstrated that the active form of folic acid, 5-methyltetrahydrofolate, improves NOS function via restoration of tetrahydrobiopterin (BH4) level, an important co-factor of NOS (Stroes et al. 2000). It is shown that folic acid directly improves BH4 and NO• bioavailability while reducing oxidant production in endothelial cells, both *in vitro* and *in vivo*. These effects are mediated by an upregulation of dihydrofolate reductase (DHFR) expression and activity (Gao et al. 2009). In the double-blind, randomized, cross-over trial, patients with DM type II, albuminuria and incipient diabetic nephropathy were treated with folic acid (5 mg/day) during 4 week period. High-dose folic acid treatment did not improve

renal endothelial function and failed to reduce albuminuria in human subjects with diabetic nephropathy (Schneider et al. 2014). Diabetic nephropathy is followed by increased serum creatinine and urea levels (Tripathi et al. 2017), so these parameters are commonly used for kidney function determination (Gowda et al. 2010). Our results showed that serum renal parameters, urea and creatinine concentrations, changed in the tested groups. Other researchers demonstrated increase in the level of serum urea and creatinine in the diabetic animals (Latifi et al. 2019; Saeed et al. 2008; de Gouveia et al. 2017; Xue et al. 2017; Chayarop et al. 2017). In our research DM did not increase the serum creatinine level, but its level was increased in the group of diabetic rats treated with folic acid. Urea concentration was increased in both diabetic groups, but it was higher in the DM + FA than in the DM group. These results may indicate possible nephrotoxic effects of folic acid. Increased homocysteine level in the FA group may be the reason of renal function damage. Homocysteine at pathological level can cause kidney injury (Hwang et al. 2008; Zhang et al. 2004). The mechanisms by which hyperhomocysteinemia affects kidneys are not completely identified. It is thought that hyperhomocysteinemia is an independent risk factor in the progression of renal disease and in the development of cardiovascular complications. Hwang et al (2011) demonstrated that hyperhomocysteinemia induces NADPH oxidase-mediated superoxide anion generation in the kidney leading to oxidative stress and that folic acid supplementation can antagonize NADPH oxidase activation through inhibition of NOX4 and p22phox expression and therefore abolish renal oxidative stress in hyperhomocysteinemic rats. In contrast to previous, the study of Jin et al. (2009) demonstrated that folic acid administered in high doses causes acute renal failure. Significant increases in creatinine and urea levels and decreased renal function were found in the offspring of diabetic progenitors and controls who received folic acid (250 mg/kg) (Corrêa et al. 2015). Renal failure caused by folic acid may be induced by the deposition of folic acid crystals, which block the tubules, or

by direct toxicity of tubular epithelial cells (Gupta et al. 2012). In rats folic acid (250 mg/kg) crystals block the renal tubules and induce their consequent acute necrosis, which is characterized by proliferation of tubular cells, infiltration of inflammatory cells and fibrosis in chronic phase (Jin et al. 2009). Therefore, in order to avoid kidney damage, during the treatment with folic acid the administered dose should be taken into account. The obtained results of our study indicate possible kidney damage induced by the folic acid treatment, although it was administered in 50 folds lower doses than in the previous studies, but the treatment lasted for longer period (28 days).

In addition to the above-mentioned complications, diabetes is followed by heart disease and cardiac remodeling. Diabetic cardiomyopathy is defined as left ventricular dysfunction, with fibrosis, inflammation, oxidative stress and metabolic disturbance in diabetic patients without coronary artery disease, hypertension or other potential etiological conditions (Varma et al. 2018; Ernande and Derumeaux 2012; Min et al. 2018). It is morphologically characterized by cardiac hypertrophy and remodeling, as well as by myocardial fibrosis (Min et al. 2018). Proteolytic enzymes, MMPs, are considered to have an important role in degradation of extracellular proteins and myocardial remodeling (Ichihara et al. 2006). These enzymes activation is found in heart failure in humans and followed by modifications in extracellular matrix structure (Ichihara et al. 2006; Thomas et al. 1998). MMPs have important role as physiological regulators of the extracellular matrix (Zhang et al. 2018). Activity change and significantly increased expression of MMP-2 and MMP-9 are associated with the onset of acute myocardial infarction (Zhang et al. 2018; Squire et al. 2004). Other authors have tested gelatinases (MMP-2 and MMP-9) activities in the cardiac tissue of diabetic experimental animals. Increased activities of both gelatinases that are associated with cardiac remodeling were obtained by Min et al. (2018), Rodriguez et al. (2006), and Bhatt and Veeranjanyulu (2012). In contrast, other studies revealed decreased MMP-2 and increased MMP-9 (Silva et

al. 2017) activities or protein concentrations (Westermann et al. 2007). Our results demonstrate that activity of proMMP-2 can be detected in all tested groups, while aMMP-2 activity was detected only in the DM group. ProMMP-2 relative activity was more than 50 % higher in the DM and FA groups than in the C group. The folic acid treatment decreased proMMP-2 activity in the diabetic rats. In this group proMMP-2 activity was increased only 10 % in comparison to the C group. Relative activity of aMMP-9 was detected in all tested groups. However, there were significant differences in MMP-9 relative activities between the groups. In comparison to the C group the aMMP-9 activity was lower in the DM group and higher in the FA and DM + FA groups. Changes in MMPs activities can be related to increased oxidative stress in diabetes (Akat et al. 2018). Kayama et al. (2015) revealed that oxidative stress worsens angiogenesis and increases fibrosis development, at most, because of modifications in activity and/or in expression of MMPs. It is reported that MMP-9 has an important role in neovascularization through the proteolytic degradation of the proteins in basal lamina of the blood vessels and a release of the biologically active form of vascular endothelial growth factor (VEGF) (Yabluchanskiy et al. 2013; Bergers et al. 2000). It is also demonstrated that folic acid is involved in angiogenesis. It exhibits the pro-angiogenic potential that is evidenced by an increased blood vessel density and branching in the chicken chorioallantoic membrane assay. The cultures treated with folic acid demonstrated spontaneous angiogenesis manifested as an increase in the number of new capillaries, vessel branching, and density (Davis et al. 2018). The aim of therapeutic angiogenesis is to promote neovascularization in ischemic tissues by targeting angiogenic growth factors such as VEGF and erythropoietin (EPO) (Chu and Wanf 2012). Folic acid treatment leads to elevated VEGF and EPO levels that might have contributed to the enhanced angiogenesis. It is suggested that folic acid supplementation enhances angiogenesis and reduces apoptosis with the increased VEGFA gene expression (Zabihi et al. 2007). Since the folic acid administration stimulates

angiogenesis, this may be the reason for the increased activity of MMP-9 in the FA group. Hou et al. (2018) demonstrated that folic acid administration reduces oxidative stress, so in these conditions the decrease in both gelatinases activities and homocysteine concentrations is expected. However, unpredictably in our study elevated homocysteine level in healthy rats treated with folic acid was obtained. Other studies showed that in hyperhomocysteinemic state there is an increase in matrix metalloproteinases activities (Miller et al. 2002; Vacek et al. 2012, Tyagi et al. 2010) that is in agreement with our findings. The increased homocysteine concentration may be the reason of increased MMP-2 activity in healthy folic acid treated rats. In contrast to previous, in the diabetic rats the folic acid treatment has positive effects since it reduced serum homocysteine concentration, as well as cardiac MMP-2 activity.

Conclusion

This study shows that STZ induced DM in rats led to numerous changes in liver, renal, and endothelial functions, as well as in the cardiac tissue during the period of four weeks. According to the obtained results, folic acid administration in diabetic rats has positive effects. It reduced significantly the blood glucose level and alleviated some of the liver damage parameters. Also its administration decreased MMP-2 activity in diabetic rats that can attenuate the progression of DM-induced cardiac dysfunction. Accordingly, we can conclude that there are cardio- and hepatoprotective effects of the folic acid administration in diabetes. Since it was demonstrated that its use can cause exacerbation of renal function parameters, the dosing of folic acid and the period of its administration should be carefully determined in order to avoid the renal damage. This represents the topic of further extensive studies. Moreover, the future research will be dedicated to investigate the potential connection between the folic acid administration and the increased homocysteine level in the healthy rats.

Conflicts of Interest

The authors declare that there is no conflict of interest associated with this work.

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Table 1. DM development confirmation serum parameters

Parameters	Groups (mean $\pm$ SEM)		P value
	C0	DM	
Glucose (mmol/l)	6.1 $\pm$ 0.2	27.7 $\pm$ 2.2	<0.001
Insulin (mU/l)	26.8 $\pm$ 1.0	20.2 $\pm$ 0.9	<0.001
HOMA-IR	7.2 $\pm$ 0.4	26.6 $\pm$ 2.2	<0.001

HOMA-IR – Homeostasis model assessment of insulin resistance

Draft

Table 2. Certain biochemical parameters in serum or plasma of the experimental animals

Parameters	Groups (mean $\pm$ SEM)				P value
	C	DM	FA	DM + FA	
Homocysteine (mmol/l)	10.4 $\pm$ 0.6	8.3 $\pm$ 0.4 *	12.9 $\pm$ 0.8 *	5.6 $\pm$ 0.4 **,□,###	<0.001
Alpha-amylase (U/l)	3911.7 $\pm$ 289.8	2084.3 $\pm$ 187.2 **	3118.7 $\pm$ 113.1 *	1575.5 $\pm$ 136.1 **,##	<0.001
Fibrinogen (g/l)	2.2 $\pm$ 0.1	1.2 $\pm$ 0.2 **	2.2 $\pm$ 0.1	2.1 $\pm$ 0.1 □□	0.004
von Willebrand factor (%d.N.)	99.4 $\pm$ 4.3	467.9 $\pm$ 43.6 **	118.7 $\pm$ 14.1	379.5 $\pm$ 35.0 **,##	<0.001

** – P < 0.01 versus C, □□ – P < 0.01 versus DM, ## – P < 0.01 versus FA, * – P < 0.05 versus C, □ – P < 0.05 versus DM.

Figure legends

Fig. 1. Effects of the folic acid treatment on body weight in non-diabetic and diabetic rats during four-week period. 1 – at the start of experimental period, 2 – first week, 3 – second week, 4 – third week, and 5 – fourth week measurement. ** – $P < 0.01$ versus C, $\square\square$ – $P < 0.01$ versus DM, $\#\#$ – $P < 0.01$ versus FA, $\#$ – $P < 0.05$ versus FA.

Fig. 2. Correlations of heart mass and body mass in the (A) C – $P = 0.036$, $r^2 = 0.666$, (B) DM – $P < 0.001$, $r^2 = 0.955$, (C) FA – $P = 0.6$, $r^2 = -0.19$, and (D) DM + FA group – $P = 0.031$, $r^2 = 0.678$.

Fig. 3. Glucose level in non-diabetic and diabetic rats during four-week period. 1 – first week, 2 – 72 h after STZ administration, 3 – second week, 4 – third week, and 5 – fourth week measurement. ** – $P < 0.01$ versus C, $\square\square$ – $P < 0.01$ versus DM, $\#\#$ – $P < 0.01$ versus FA, $\square$ – $P < 0.05$ versus DM.

Fig. 4. The activities of serum aspartate aminotransferase – AST (A), alanine aminotransferase – ALT (B), and alkaline phosphatase – ALP (C) in non-diabetic and diabetic rats. ** – $P < 0.01$ versus C, $\square\square$ – $P < 0.01$ versus DM, $\#\#$ – $P < 0.01$ versus FA.

Fig. 5. Serum total protein and albumin concentrations in non-diabetic and diabetic rats. ** – $P < 0.01$ versus C, $\#\#$ – $P < 0.01$ versus FA, $\square$ – $P < 0.05$ versus DM.

Fig. 6. Serum urea and creatinine concentrations in non-diabetic and diabetic rats. ** – $P < 0.01$ versus C, $\square\square$ – $P < 0.01$ versus DM, $\#\#$ – $P < 0.01$ versus FA, $\square$ – $P < 0.05$ versus DM.

Fig. 7. Representative zymograms of cardiac tissue matrix metalloproteinase (MMP-2 and MMP-9) activities detected by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (A); relative activities of MMP-2 and MMP-9 in non-diabetic and diabetic rats.

aMMP-9 – active form of matrix metalloproteinase-9; proMMP-2 – latent form of matrix metalloproteinase-2; aMMP-2 – active form of matrix metalloproteinase-2. ** – $P < 0.01$ versus C, $\square\square$ – $P < 0.01$ versus DM, $\#\#$ – $P < 0.01$ versus FA.

130x81mm (300 x 300 DPI)

254x211mm (300 x 300 DPI)

128x81mm (300 x 300 DPI)

100x92mm (300 x 300 DPI)

254x127mm (300 x 300 DPI)