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ORIGINAL ARTICLE

Influence of vitamin C or/and L-methionine on hyperglycaemia, hyperlipidaemia and hematological alterations in alloxan-induced diabetes in rats

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Summary. Background: Here, we assessed the possible therapeutic potential of vitamin C or/and L-methionine on some biochemical and hematological alterations in alloxan-induced diabetes in rats. Diabetes was induced by single intraperitoneal (100 mg/kg b.w, i.p) dose of alloxan monohydrate solution. The animals were randomly grouped into five as follows: the normal control group, diabetic control group, diabetic treated with vitamin C (Vit C) group, diabetic treated with L-methionine (L-Meth) group, diabetic treated with Vit C and L-Meth group. The regimens were given once daily for four weeks. Significant disturbances in glucose, glycated hemoglobin, lipid profiles, prothrombin time (PT), partial thromboplastin time (PTT), some hematological and oxidant/antioxidant parameters was observed in diabetic control rats. Only the combination of Vit C and L-Meth had the most beneficial effect on hyperglycemia, dyslipidemia, abnormal coagulation indices and oxidative stress in alloxan-induced diabetes in rats. From the present data, we concluded that Vit C and L-Meth in combination may have therapeutic effects against the risks of the metabolic syndrome and coronary artery diseases and could improve the health of rats with diabetes mellitus.

Key words: Antioxidant, L-methionine, oxidative stress, partial thromboplastin time, prothrombin time, vitamin C

Introduction

Diabetes mellitus (DM) is related to a group of complex metabolic disorders including hyperglycemia, dyslipidemia, hypercoagulability, thrombosis and impaired protein metabolism that provoke many complications such as coronary artery diseases (CAD), neuropathy and cardiomyopathy (1). Alloxan induced diabetes represent a good model for the study of insulin-dependent DM (type 1). Oxidative stress results mainly due to the production of free radicals and a deficiency in antioxidant defense mechanisms (2). It could play an important role in the development/progression of diabetic tissue damage and increased lipid peroxidation (3,5). Biomarker of oxidative stress is recognized as a significant mediator in the development of cardiovascular complication in DM, as well as the potential for prevention of complications through the use of antioxidants supplementation.

Vit C (Vit C) is a potent reducing agent and prevent oxidative stress by inhibiting free radicals production. The antioxidant effects of Vit C could be attributed to its reversible oxidation and reduction characteristics (2,6). Oxidized form of Vit C anticipates in redox recycling of Vit E (7). Methionine is an essential α-amino acid in humans (8). It found in two enantiomers (L- methionine and D-methionine). L-Meth is the active form. It serves as a precursor of glutathione synthesis via its conversion to cysteine by the transsulfuration pathway. Also, it is important in the maintenance of nitrogen balance, the metabolism/regulation of nucleic acids and for the structure/function of membranes and necessary for re-methylation processes. Also, L-Meth has an active metabolite S-adenosyle methionine (SAM) which has a significant role as anti-oxidant. SAM neutralizes free radicals' toxicity by methylation and transsulfuration that might con-

tribute its role in modulating central nervous system oxidative stress (9). Besides that, up to now, only few reports have appeared concerning Vit C and L-Meth and hemostasis. DM is associated with vascular abnormalities and disturbances in hemostasis that could contribute to the development of thrombotic complications. Prothrombin time (PT) and partial thromboplastin time (PTT) are hematological indices that give an insight into the coagulation status of patients (10). Therefore, the present study was designed to investigate the hypothesis that the administration of an antioxidant Vit C or/and L-Meth modulates alloxan induced oxidative stress on some biochemical and hematological parameters in diabetic albino Wistar rats.

Materials and methods

Chemicals

Alloxan monohydrate ($C_4H_2N_2O_4 \cdot H_2O$; molecular weight 160·08 Da) was purchased from Sigma-Aldrich (St Louis, MO, USA). Vit C tablets and L-Meth powder were purchased from Adwia Pharmaceuticals and El-Gomhouria Company for Trading Chemicals and Medical Appliances, respectively (Cairo, Egypt).

Experimental animals

Thirty-five adult male Wistar albino rats (*Rattus norvegicus*), weighing 100–120 g, were used in the current study. The animals were purchased from the Vaccine and Serum Experimental Animals Production Center in Giza, Egypt. Animals were housed in suitable plastic cages and acclimatized to laboratory conditions for a period of 1 week before the commencement of the experiments under good ventilation condition. Rats were given *ad libitum* access to water and food pellets (Agricultural- Industrial Integration Company, Giza, Egypt). All animals were humanely treated in accordance with the WHO guideline for animal care and the study design was approved by the Ain Shams University Research Ethics Committee.

Induction of DM

Induction of type-1 DM in rats was induced by single i.p injection of alloxan (100 mg/kg i.p) dissolved in normal saline (11). To prevent initial fatal hypoglycemia, each rat was allowed to drink 5% glucose solution for twenty-four hours. After three days, successful induction of DM in rats was established by measuring the fasting blood glucose using electronic digital glucometer (the range above 200 mg/dl). These diabetic rats were selected for the further study.

Experimental design

The experiments animals were divided randomly into five groups of seven rats each.

Group I: served as normal control group; Group II: Alloxan (100 mg/kg b. w.) induced diabetic control; Group III: Alloxan induced diabetic rats treated for four weeks with daily and orally of Vit C (200 mg/kg b.w) as recommended by (12); Group IV: Alloxan induced diabetic rats treated for four weeks with daily and orally of L-Meth (50 mg/kg b.w) as recommended by (13); Group V: Alloxan induced diabetic rats treated for four weeks with Vit C plus L-Meth.

At the end of the experiment, the animals of all groups were anesthetized with light diethyl ether then sacrificed and the blood samples were obtained.

Blood sampling

The blood samples were collected into clean test-tubes with or without EDTA (Ethylenediaminetetraacetic acid) and with sodium citrate solution. A portion of blood with EDTA was used to determine some hematological parameters. Another blood portion without EDTA was left to coagulate at room temperature (37°) and the clotting time was recorded using a stopwatch. Then, blood was centrifuged in a cooling centrifuge to separate serum, which was divided into samples and preserved at –80°C. Other blood portion with sodium citrate solution was used to determine PT and PTT.

Biochemical estimations

Serum glucose and glycated hemoglobin (HbA1c) was estimated by previous methods (14,15). Serum total cholesterol, triglyceride, high density lipoprotein cholesterol (HDL-cholesterol) and low-density lipoprotein cholesterol (LDL-cholesterol) were determined (16-19). Very low-density lipoprotein cholesterol (VLDL-cholesterol) was calculated using the equation: VLDL= TC – (HDL + LDL). Atherogenic Index of Plasma, Castelli Risk Index (CRI) and Ath-

erogenic Coefficient are the three ratios we studied in predicting the risk of CAD. These ratios were calculated according to the following equations: Atherogenic Index of Plasma = (log Triglyceride /HDL-cholesterol), Castelli's Risk Index (CRI-I) = (Total cholesterol / HDL-cholesterol), Castelli's Risk Index (CRI-II) = (LDL-cholesterol / HDL-cholesterol), Atherogenic Coefficient = (Triglyceride – HDL-cholesterol)/ (HDL-cholesterol). Catalase (CAT), glutathione peroxidase (GPx), glutathione reduced (GSH), superoxide dismutase (SOD) activities, and thiobarbituric acid reactive substances (TBARS) level were determined using commercially available kits following the instructions of the manufacturer (Bio-diagnostic, Giza, Egypt).

Hematological estimations

Total red blood cell (RBC), hemoglobin (Hb), hematocrit value (HCT) count and blood indices (mean cell volume; MCV, mean corpuscular hemoglobin; MCH and mean corpuscular hemoglobin concentration; MCHC) were measured by Coulter (Hemat 8 analyser; SEAC, Freiburg, Germany). PT and PTT were estimated according to previous studies (20, 21). Mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated as outlined by Dacie and Lewis (18). PT and PTT were estimated according to previous studies (20, 21).

Statistical analyses

Data are presented as mean values with their standard errors. Statistical analysis was performed with One Way Analysis of Variance (ANOVA) and the differences among groups were determined by Tukey's multiple comparison test (Turner and Thayer, 2001) using GraphPad Prism version 4.03 for Windows (GraphPad Software Inc., San Diego, CA, USA). Statistically significant variations were compared as follows: (a) all groups *v.* the control group; (b) alloxan plus Vit C and/or L-Meth treated groups *v.* the alloxan-only treated group; (c) alloxan plus Vit C and/or L-Meth treated groups *v.* alloxan plus Vit Cand/or L-Meth treated groups (with each other). P values of <0·05, < 0·01 and < 0·001 were considered significant, highly and very highly significant, respectively.

Results

Vit C and/or L-Meth alleviated changes in biochemical parameters of alloxan-induced diabetic rat model

As shown in Fig. 1, serum glucose level and HbA1c percent were significantly increased (P < 0.001) in the alloxan only compared with the control group. Moreover, treatment of rats with alloxan plus Vit C and/or L-Meth resulted in significant reduction (P < 0.001) in serum glucose level and HbA1c compared with the alloxan only group. The utmost modulation of serum glucose level and HbA1c percent was brought by the combined treatment with Vit C and L-Meth. Furthermore, a significant difference in serum glucose level and HbA1c (P < 0.001) was found between the treated groups with either Vit C or L-Meth compared with the combined treatment with Vit C and L-Meth.

All these results revealed that the most modulatory effects on diabetic rats were induced by the combined treatment with Vit C and L-Meth.

The present study showed that total cholesterol, triglycerides, LDL-cholesterol, VLDL-cholesterol, Castell's risk index I & II, atherogenic index of plasma and atherogenic coefficient were significantly increased (P < 0.001) with a concomitant decrease in HDL-cholesterol (P < 0.001) in the alloxan only compared with the control group (Fig. 2).

Treatment of rats with alloxan plus Vit C and/or L-Meth produced significant decrease (P < 0.05-0.001) in total cholesterol, triglycerides, VLDL-cholesterol, Castell's risk index I, atherogenic index of plasma and atherogenic coefficient compared with the alloxan only group. In addition, treatment of rats with alloxan plus Vit C or alloxan plus Vit C and L-Meth resulted in significant increase (P < 0.05- 0.001) in HDL-cholesterol compared with the alloxan only group, while treatment of rats with alloxan plus L-Meth did not significantly change (P > 0.05) in HDL-cholesterol compared with the alloxan only group. On the other hand, treatment of rats with alloxan plus vitamin C and L-Meth resulted in significant decrease (P < 0.05-0.001) in LDL-cholesterol and Castell's risk index II compared with the alloxan only group, while treatment of rats with alloxan plus Vit C or L-Meth did not significantly change (P > 0.05) in LDL-cholesterol and Castell's risk index II compared with the alloxan only

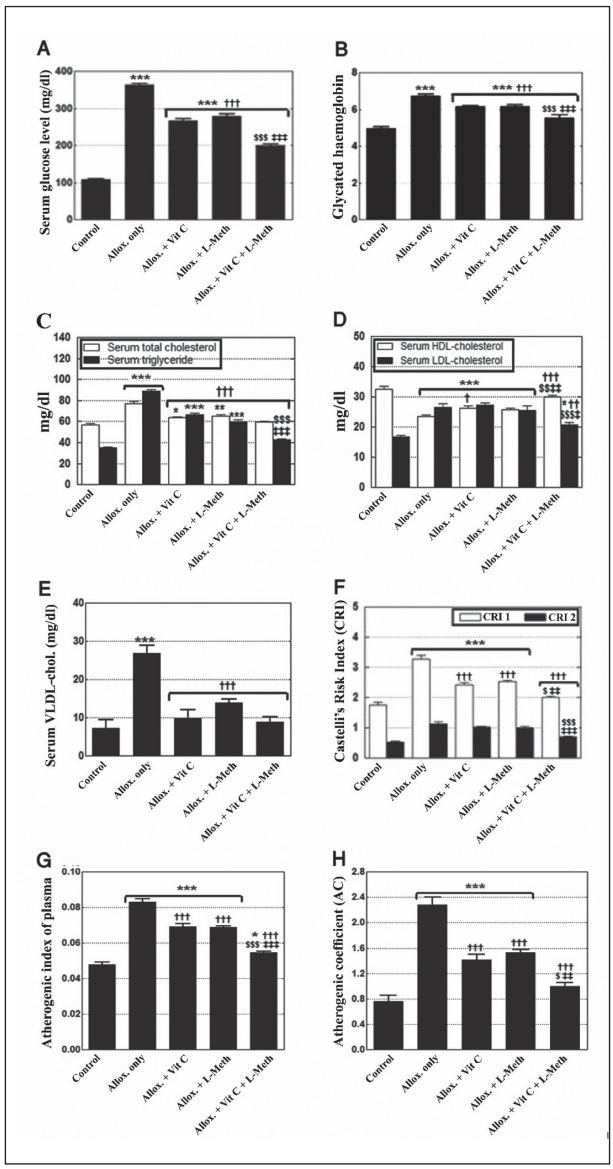

Figure 1. Biochemical parameters of control and diabetic rats. Serum glucose level (A), glycated haemoglobin (B), total cholesterol & triglyceride (C), HDL-cholesterol & LDL-cholesterol (D), VLDL-cholesterol (E), Castell's risk index I& II (F), atherogenic index of plasma (G) and atherogenic coefficient (H). Values are means, with their standard errors represented by vertical bars. Allox., Alloxan; Vit C, vitamin C; L-Meth, L-methionine.

Mean values were significantly different from that of the control group: *P < 0.05, ***P < 0.001.

Mean values were significantly different from that of the Allox. only group: †P < 0.05, †††P < 0.001.

Mean values were significantly different from that of the Allox. plus Vit C group: $ P < 0.05, $$ P < 0.01, $$$ P < 0.001.

Mean values were significantly different from that of the Allox. plus L-Meth group: ‡ P < 0.05, ‡‡ P < 0.01, ‡‡‡ P < 0.001.

group. Furthermore, a significant difference was found between the treated groups compared with the combined treatment with Vit C and L-Meth.

All these results revealed that the maximum improvement of lipid profile, atherogenic indexes and coefficient was brought by the combined treatment with Vit C and L-Meth. Also, treatment of diabetic rats with combination of Vit C and L-Meth restore the total cholesterol, triglycerides, VLDL-cholesterol, HDL-cholesterol, Castell's risk index I & II and atherogenic coefficient to normal (P >0.05, compared with the control group).

Vit C and/or L-Meth alleviated changes in hematological parameters of alloxan-induced diabetic rat model

The present study showed that blood erythrocytes (RBC), hemoglobin (Hb) content and hematocrit value (HCT) were significantly decreased (P < 0.05–0.001) in the alloxan only compared with the control group (Fig. 2), while the mean corpuscular volume (MCV), mean corpuscular Hb (MCH) and mean corpuscular Hb concentration (MCHC) did not significantly change (P > 0.05) in the alloxan only group compared with the control group. All these hematological changes were significantly modulated (P < 0.05–0.001) in the group treated with alloxan plus Vit C and/or L-Meth compared with the alloxan only group.

All these hematological changes were reverted to near normal levels (P > 0.05, compared with the control group) in the group treated with alloxan plus Vit C and L-Meth, since there was statistical difference (P < 0.05–0.001) recorded between the group treated with alloxan plus Vit C and L-Meth. These results revealed that the highest improvement of the above parameters was brought by the combined treatment with Vit C and L-Meth.

As shown in Fig. 2, treatment of rats with alloxan alone showed significant increase (P < 0·05) in PT and PTT compared with the control animals. On the other hand, treatment of rats with alloxan plus Vit C and/or L-Meth produced significant decrease (P < 0·01-0·001) in PT and PTT compared with the alloxan only group. Moreover, a significant difference (P < 0·05) in PT was found between the treated groups with either Vit C or L-Meth compared with the combined treatment with Vit C and L-Meth.

All these results revealed that the most modulatory effects on diabetic rats were induced by the combined treatment with Vit C and L-Meth. In addition, there is no significant difference was recorded between alloxan plus Vit C and alloxan plus L-Meth.

Vit C and/or L-Meth alleviated changes in serum oxidative stress markers of alloxan-induced diabetic rat model

Table 1 revealed that serum TBARS was significantly higher (P < 0·001) in the alloxan only group compared with the control animals. On the other hand, treatment of rats with alloxan alone showed significant decrease (P < 0·001) in GSH level, SOD, GPx, and CAT activities compared with the control animals. Oral treatment of diabetic rats with Vit C and/or L-Meth were significantly (P < 0·001) improving these parameters compared with the alloxan only group. Furthermore, a significant difference (P< 0·001) in serum TBARS and enzymatic/non-enzymatic antioxidant (P < 0.01- 0.001) was found between the treated groups with either Vit C or L-Meth compared with the combined treatment with Vit C and L-Meth.

Discussion

Alloxan generates reactive oxygen species (ROS), which cause the selective destruction of pancreatic

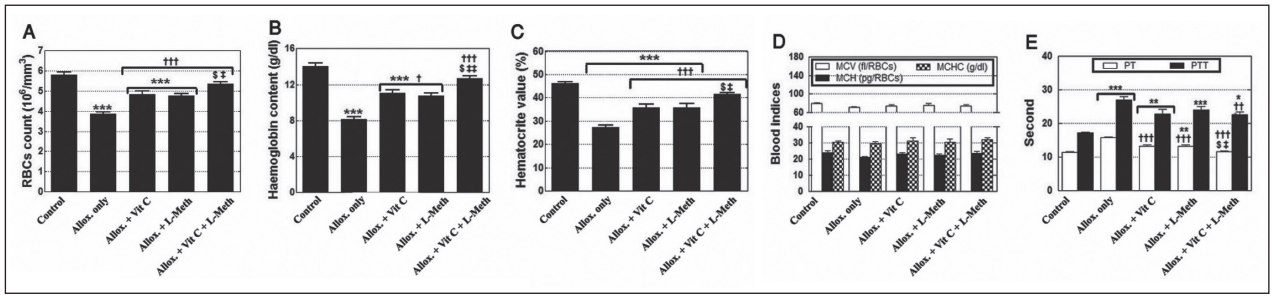

Figure 2. Hematological parameters of control and diabetic rats. RBC count (A), hemoglobin content (B), hematocrit value (C), blood indices (D), and prothrombin time (PT), partial thromboplastin time (PTT) (E). Values are means, with their standard errors represented by vertical bars. MCV: The mean corpuscular volume, MCH: mean corpuscular Hb, MCHC: mean corpuscular Hb concentration and RBC: red blood corpuscle. Blood indices did not significantly change (P > 0.05) among all groups. Allox., Alloxan; Vit C, vitamin C; L-Meth, L- methionine.
Mean values were significantly different from that of the control group: *P < 0.05, ***P < 0.001.
Mean values were significantly different from that of the Allox. only group: †P < 0.05, †††P < 0.001.
Mean values were significantly different from that of the Allox.plus Vit C group: $ P < 0.05, $$ P < 0.01, $$$ P < 0.001.
Mean values were significantly different from that of the Allox.plus L-Meth group: ‡ P < 0.05, ‡‡ P < 0.01, ‡‡‡ P < 0.001.

Table 1. Effect of Vit C and/or L-Meth on serum oxidative stress markers (TBARS, GSH, SOD, GPx, CAT) of alloxan-induced diabetic rat model

	Groups				
Parameters	**Control**	**Allox. only**	**Allox. + Vit C**	**Allox. + L-Meth**	**Allox. + Vit C + L-Meth**
TBARS level (mmol/mL)	21.12±0.40	39.76±0.58***	31.80±0.80***†††	35.67±0.98***†	26.61±0.90***††† $$$‡‡‡
GSH level (mg/dL)	51.92±0.62	23.62±1.04***	40.65±0.59***†††	36.41±1.70***†††	46.95±1.29*††† $$‡‡‡
SOD activity (U/mL)	160.65±0.36	80.84±0.10***	130.21±0.19***†††	110.56±0.16***†††	150.10±0.25***††† $$$‡‡‡
GPx activity (U/mL)	141.10±0.85	94.06±1.02***	119.90±1.53***†††	112.30±1.91***†††	129.70±0.70***††† $$$‡‡‡
CAT activity (U/mL)	241.40±1.51	139.80±1.21***	201.50±0.72***†††	189.00±2.39***†††	225.70±1.76***††† $$$‡‡‡

CAT: catalase, GPx: glutathione peroxidase, GSH: glutathione reduced, SOD: superoxide dismutase, TBARS: thiobarbituric acid reactive substances Values are means, with their standard errors. Allox., Alloxan; Vit C, vitamin C; L-Meth, L- methionine.
Mean values were significantly different from that of the control group: *P < 0.05, ***P < 0.001.
Mean values were significantly different from that of the Allox. only group: †P < 0.05, †††P < 0.001.
Mean values were significantly different from that of the Allox.plus Vit C group: $ P < 0.05, $$ P < 0.01, $$$ P < 0.001.
Mean values were significantly different from that of the Allox.plus L-Meth group: ‡ P < 0.05, ‡‡ P < 0.01, ‡‡‡ P < 0.001.

β-cells and significant hypo-insulinemia (type 1 DM) that is concomitant with hyperglycemia, dyslipidemia and impairment of hematological parameters (anemia), coagulation indexes (PT and PTT), endogenous antioxidant system, and alteration in lipid peroxidation as reported here (4, 10, 22-29). Hyperglycemia exposes RBCs leads to glycation of hemoglobin, prothrombin, fibrinogen and other proteins involved in clotting mechanisms. These glycation results in the incomplete activation/function of the clotting cascade. Glycation of intrinsic/extrinsic clotting proteins will decrease the availability of these proteins which affect the clotting capacity (10). PT and PTT are important coagulation parameters (10). PT is used to detect important deficiencies (and rarely inhibitors) of factors I (fibrinogen), II (prothrombin), V, VII, and X of the extrinsic and common pathways. PTT is used to detect bleeding disorders due to deficiencies of factors II, V, VIII, IX, X, XI, and XII of the intrinsic and common pathways (26). Prolongation plasma levels of PT and/or PTT indicates a problem with the quantity and/or quality of single or multiple factors within the relevant pathways. In patients with DM, the elevation of these parameters (PT and PTT) are consistent with abnormal coagulation cascade, fibrinolytic system and platelet dysfunction; and may be interpreted as a tendency to bleeding and/or cardiovascular disease (CVD). Hypertriglyceridemia in DM, the additional risk factor for CVD, in addition to elevated levels of tissue factor (III) and an activated factor (VII_a) gives a bad prognosis for serious fatal ischemic heart disease (10, 27). Hyperglycemia has been the causative factor of these abnormalities in coagulation pathways. In addition, it exposes patient with DM to macrovascular mortality (27). Numerous studies have demonstrated that levels of PT are increased in either type 1 or type 2 DM (10, 26, 27).

The current finding showed that the combined treatment of Vit C and L-Meth were most effective in modulating hyperglycemia, hyperlipidemia, hematological abnormalities, and decreasing lipid peroxidation and oxidative stress and may also modify coagulation and fibrinolysis (2,3,8, 12, 30, 31). The hypoglycemic effect of Vit C may be linked to its antioxidant effect on β cells (32). Another explanation proposed for reduction of blood glucose level is that Vit C-mediated increase in insulin action is mainly due to an improvement in non-oxidative glucose metabolism. Some studies showed that a decrease in plasma Vit C causes hyperlipidemia and hypertension in DM (33, 34). Also, the antioxidant properties of Vit C seem to be effective against different conditions related to promotion of CAD such as high blood pressure, impaired glucose and lipid profile, endothelial dysfunction and LDL-cholesterol oxidation (35). Vit C may affect the clearance rate of lipoproteins by reducing the glycosylation and peroxidation of apolipoproteins such as apoE for LDL-receptors with apoC- III, apoA-I for HDL receptors (36, 37). The improvement of glycemic control as well as lipid profiles done by L-Meth was mainly due to increase and decrease glutathione concentration and oxidative stress, respectively, that serve as antioxidant agent (30, 38, 39, 40). Another possible explanation could be proposed that L-Meth may act as a synergist with Vit C by increasing Vit C levels through the elevated reduction of dehydroascorbic acid to ascorbic acid (13, 38,39). Another study suggested that the significant decrease which was noted in the HbA1c (HbA1c) in patients supplemented with Vit C could be attributed to the competition of Vit C with glucose for the reaction with amino groups on the hemoglobin beta chain (41-44). L-Meth could serve as ROS scavenging system to protect proteins from oxidation such as HbA1c and may improve RBCs count and Hb content as well as HCT value perhaps by reducing the protein glycosylation and increasing glutathione (40, 41, 45). Thus, the combined treatment with Vit C plus L-Meth may decrease the glycation of protein and therefore it may reduce the glycation and oxidation of prothrombin and other coagulation factors leading to decreasing their removal from the circulation. L-Meth and its residues can act as powerful antioxidants (47-49). Because its oxidation to Meth sulphoxide (MSO) is reversible. MSO can be reduced back to Meth by Meth sulphoxide reductase (MsrA) with thioredoxin serving as electron donor (50-52). Thus, L-Meth, along with the reduction/oxidation glutathione system (GSH/GSSG), forms remarkable link in the antioxidant defense system and contributes to the pro-oxidant/antioxidant homeostasis within the cell (9). In a previous study, it was reported that Vit C had an affirmative effect on the oxidative stress

in plasma and tissue (53, 54). Vit C acts as scavenger for ROS and RNS, thus effectively protecting other substrates from oxidative damage in health and disease like DM (53, 55,56). Other studies reported that Vit C inhibits lipid peroxidation of RBCs membrane, increases Hb concentration by inhibiting oxidation of the tetrahydrofolates and improves erythropoietic activity and glutathione concentration (13, 57, 58). It has been postulated that L-Meth inhibited a hypotonic hemolysis in rats through its action as a membrane stabilizing agent and induced a significant increase in RBCs count (13). This may be related to their effect on RBCs membrane integrity where Vit C and L-Meth may act as a membrane stabilizing agent (13, 58). Moreover, Vit C plays the main role in the antioxidant defensive system, conserving all lipids subjecting into oxidation (34, 59, 60). It has been suggested that Vit C can regenerate the oxidized Vit E (the lipid-soluble Vit) by reducing α-tocopheroxyl radicals in membranes (5). Vit E acts as a powerful terminator of lipid peroxidation and improves glutathione system (34, 61) that may stabilize the integrity of the cell membrane and keeping the membrane intact and the enzymes enclosed through scavenging free radicals. Furthermore, Vit E is very effective in glycemic control, lowering HbA1c level and preventing the hypertrophic effects of hyperglycemia (61). Meth can also be a part of the regeneration of the α-tocopherol radical (8). The protective role of free radical scavenger is by the hydrogen donor ability of tocopherol. Another major biological role of Vit E and α- tocopheryl inhibit platelet aggregation (53). Meth influences the metabolism of elements participating in the biosynthesis of SOD (64). However, Patra et al. (65) reported that administration of rats with L-Meth may lead to an increased activity of SOD in the liver. In addition, SOD plays a major role in anti-oxidant defense that dismutates superoxide radicals to oxygen and hydrogen peroxide.

Therefore, the possible hypoglycemic, hypolipidemic and antianemic mechanisms of Vit C plus L- Meth may be due to their antioxidant properties through transferring electrons and free radicals or chelating metals catalyst and activating antioxidant system.

Conclusion

Vit C plus L-Meth supplementation might provide a simple, safe, and more effective means for preventing and ameliorating chronic complications of DM than each given alone perhaps because their action as antioxidant beside their important role against the risks of the metabolic syndrome and coronary artery diseases. Therefore, further studies are required to validate therapeutic effects of these antioxidants in various experimental models.

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