Effect of oral methionine on blood lipid peroxidation and antioxidants in alloxan-induced diabetic rats

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Supplementation of thiol compounds has been suggested to protect against the toxic effects of reduced oxygen species by contributing to the thiol pool of the cell. The present study was designed to determine whether supplementation of methionine in the diet of diabetic animals protected against the oxidative stress in diabetic pathology. Oral methionine was administered at a dosage of 330 mg/100 g feed to diabetic rats. The effect was compared with the effect of insulin administration. Levels of lipid peroxides were measured in plasma, erythrocytes, and erythrocyte membrane. Anti-oxidants were measured in plasma. Diabetic condition was associated with increased lipid peroxidation and depletion in antioxidant levels. Although methionine did not affect the level of blood glucose and some of the antioxidants, it lowered the lipid peroxide content in blood. Erythrocyte lipid peroxidation activity was unaffected by methionine treatment. Administration of insulin lowered both plasma and erythrocyte lipid peroxide levels.

Keywords: methionine; diabetes mellitus; lipid peroxidation; antioxidants

Introduction

Active oxygen metabolism plays a role in the normal functioning of the β-cells of the pancreas. Several studies carried out in recent years show increased lipid peroxidation in diabetes. For example, increased circulating lipid peroxides are encountered in the blood of diabetic patients[1] and experimental diabetic mice.[2] The level of lipid peroxides in the tissues of diabetic rats are shown to be high.[3] Circulating lipid peroxides are shown to be capable of initiating atherosclerosis[4] which is a well-known late feature of diabetes. It is also shown that rabbits injected with linoleic acid hydroperoxide have aortic lesions.[5]

The level of lipid peroxidation in cells is controlled by various cellular defense mechanisms consisting of enzymatic and nonenzymatic scavenger systems.[6,7] The levels of these defense mechanisms are altered in diabetes[8] and, therefore, the ineffective scavenging of free radicals may play a crucial role in determining tissue injury.

Thiol compounds are well known for their free radical scavenging property.[9] Dietary supplementation of sulphur-containing amino acids has been reported to improve the tissue antioxidant status of rats,[10] and methionine has been shown to provide a significant source of sulphur incorporated into intracellular glutathione.[11] Furthermore, the administration of supplementary methionine to rats treated with lanthanum chloride and neodymium chloride offers a protective effect against metal intoxication.[12]

No previous study has been done on the effect of dietary methionine on the lipid peroxidation reactions in diabetes mellitus. In the present study, we administered methionine to diabetic rats and determined the role of feeding methionine on blood lipid peroxidation and antioxidant levels. The effect was compared with that of untreated and insulin-treated diabetic rats.

Materials and methods

Adult male rats of Wistar strain weighing 150 to 200 g were used for the study. The animals were provided with food and water ad libitum. For the induction of diabetes, the animals were fasted overnight and di-

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vided into two groups. The experimental group of rats was injected with alloxan monohydrate (100 mg/kg body wt) intraperitoneally. Alloxan was dissolved freshly in physiological saline and was injected in 0.5 ml volumes. The control group of rats received 0.5 ml of physiological saline intraperitoneally. The diabetic state was confirmed 2 days after alloxan injection by weight loss, glucosuria (Benedict's test), and hyperglycemia (*o*-toluidine test). From the third day of alloxan injection, two sets of diabetic animals were treated with insulin or methionine for a period of 15 days. Accordingly, five groups of animals were maintained as follows:

Group I—Normal rats, injected with 0.5 ml of physiological saline, formed the control group.
Group IIa—Diabetic rats maintained on commercial rat chow for 15 days.
Group IIb—Diabetic rats maintained on commercial rat chow and injected with protamine zinc insulin (2 units/100 g body weight) daily, intraperitoneally, for a period of 15 days from the third day of alloxan injection.
Group IIc—Diabetic rats maintained on the commercial rat chow supplemented with L-methionine (330 mg/100 g feed) for a period of 15 days.
Group III—Normal rats maintained on the commercial rat chow supplemented with L-methionine (330 mg/100 g feed) for a period of 15 days.

Blood sugar levels were determined periodically by the method of Sasaki and Matsui,[13] and the body weights of animals were recorded every third day throughout the study.

At the end of 15 days, the animals were fasted overnight, killed by cervical dislocation, and blood was collected with EDTA as anticoagulant. Blood was centrifuged for 15 minutes at 3,000 rpm to separate plasma. The packed cells were washed several times with physiological saline. The buffy coat was removed and the erythrocytes were used for the study. An aliquot of the erythrocyte suspension was used for the preparation of erythrocyte membrane according to the method of Dodge et al.[14]

Hemoglobin,[15] methemoglobin,[16] and glutathione[17] (GSH) were estimated in whole blood. Plasma was analyzed for lipid peroxides,[18] α-tocopherol,[19] ascorbic acid,[20] and ceruloplasmin.[21] Lipid peroxidation studies in erythrocyte and erythrocyte membrane were carried out as described by Cynamon et al.[22] Osmotic fragility test was performed following the method of Parpart et al.[23] Erythrocyte membrane protein was measured according to the method of Lowry et al.[24]

Results

The untreated diabetic animals had the fasting blood glucose level of 201.9 ± 29.5 mg/dl, while control animals showed 77.0 ± 9.2 mg/dl. Insulin-treated animals showed significantly lower fasting glucose levels (106.8 ± 8.0 mg/dl) when compared to diabetic animals. Methionine feeding did not reduce the blood glucose levels of diabetic animals and was found to be 182.2 ± 17.1 mg/dl. The body weight changes in control and experimental animals are shown in *Figure 1*. The mean initial body weight of each group was kept between 230 g to 238 g. The control and methionine fed animals (Groups I and III) registered a significant weight gain while the untreated diabetic animals (Group IIa) showed a progressive reduction in body weight. The final body weights of diabetic animals (210 ± 3.5 g) were significantly lower than that of controls (242.1 ± 4.6 g). Insulin-treated diabetic animals (Group IIb) showed a gain in weight during experimental period compared to diabetic animals, but they never reached the weight of the controls. However, methionine-treated diabetic animals (Group IIc) did not gain weight during the experimental period.

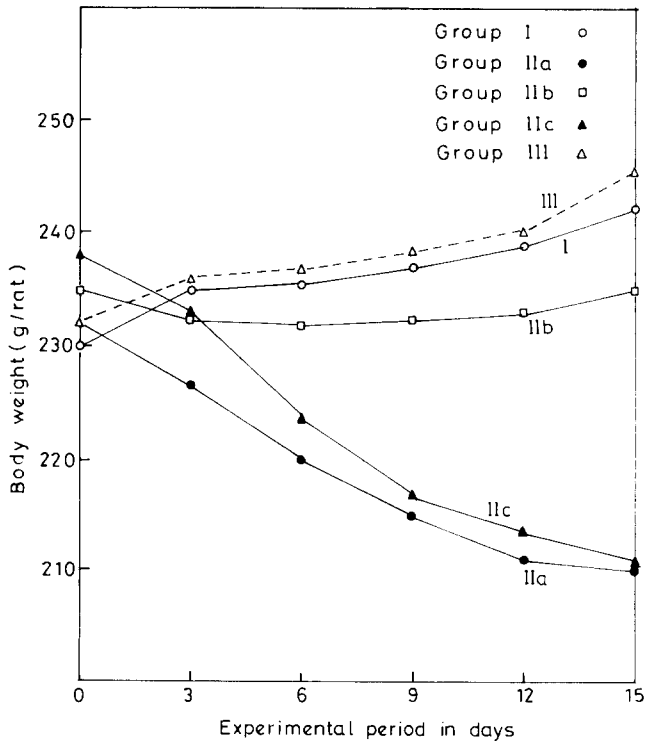

Figure 1 Body weights (mean ±SE) of control and experimental animals (n = 6). On day 0, controls were injected with physiological saline, and rats to be made diabetic received alloxan in physiological saline. Treatments with insulin or methionine were begun on day 3.

The osmotic fragility curves are shown in *Figure 2*. The value of mean corpuscular fragility in terms of saline concentration in the blood of diabetic animals was significantly high when compared to that of control animals (Control: 0.57%; diabetic: 0.68%). The initial hemolysis was observed at the concentration of 0.64% sodium chloride for control, while for diabetic and hemolysis occurred at 0.75% sodium chloride. The MCF values for insulin-treated and methionine-treated animals were 0.60 ± 0.02 and 0.69 ± 0.03, respectively.

The fragility curve of control animals had a sigmoid shape and was symmetrical. In diabetic animals, there

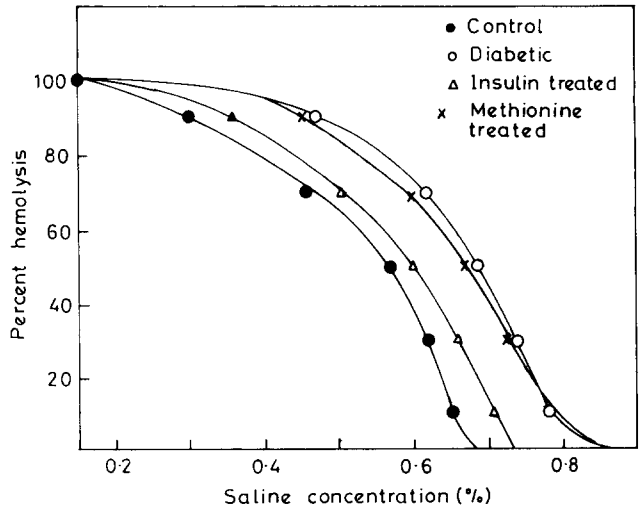

Figure 2 Osmotic fragility curves of control and experimental animals. The fragility curve of methionine treated diabetic animals was similar to that of diabetic animals.

was also a shift towards the right of the body of the fragility curve. The fragility curve of the insulin-treated animals lay between those of control and diabetic animals. The methionine-treated diabetic animals showed increased osmotic fragility similar to that of untreated diabetic animals.

Plasma lipid peroxide level was found to be significantly high in diabetic animals ($P < 0.01$) compared to that of control (*Table 1*). The elevated lipid peroxide levels observed in the blood of diabetic rats were lowered by insulin administration and also by oral methionine treatment.

Gross changes in blood hemoglobin levels and antioxidant activities were observed in diabetic animals compared to control animals (*Table 1*). Blood hemoglobin levels were found to decrease significantly ($P < 0.001$) in diabetic animals, and the level was found to be restored by insulin treatment. Methionine treatment had no effect on the low levels of blood hemoglobin observed in diabetic rats. Methemoglobin level was found to be within the normal range of 1 to 2% in both control and experimental animals.

Blood glutathione was significantly low, (22.9 ± 1.5 mg/dl) in diabetic animals compared to control animals (28.7 ± 1.7 mg/dl). Treatment of diabetic animals with insulin or methionine restored glutathione levels to near normal value. The mean ascorbic acid level was reduced markedly in diabetic animals when compared to that of control animals (control, 1.67 mg/dl; diabetic, 1.29 mg/dl). There was only a partial restoration of the ascorbic acid level by treatment with either insulin or methionine. α-tocopherol levels were significantly elevated in diabetic animals ($P < 0.001$) compared to control animals. Insulin treatment reversed this and the value was brought to normal. Ceruloplasmin level was also significantly raised in diabetic animals ($P < 0.001$). The value remained elevated in both insulin- and methionine-treated animals.

Tables 2 and *3* summarize the activities of lipid peroxidation under different incubation conditions in the erythrocytes and erythrocyte membranes of control and experimental animals, respectively. It is evident that in both diabetic and methionine-treated diabetic animals, the activity of lipid peroxidation was significantly higher than that of control under all incubation conditions. Increased lipid peroxidation was observed upon incubation of erythrocyte and erythrocyte membrane with 3% H_2O_2. Lipid peroxidation activity was maximum in the presence of 0.75% H_2O_2 and sodium azide. The percent maximal release for erythrocytes and erythrocyte membrane were 35.8 and 21.3 for control animals and 53.2 and 36.5 for diabetic animals, respectively. *Table 4* gives the levels of lipids in the plasma of control and experimental animals. Total and individual lipid levels were significantly increased in diabetic animals. Insulin treatment restored the lipid alterations in the diabetic rats while methionine treatment did not alter the lipid levels.

Discussion

Hemoglobin levels are decreased in diabetic animals while methemoglobin levels are unaltered. Osmotic fragility is increased in the erythrocytes of diabetic animals and treatment with insulin restores near to the

Table 1 Blood hemoglobin, methemoglobin, glutathione, ascorbic acid, plasma lipid peroxides, ceruloplasmin, and α-tocopherol levels in control and experimental animals. Values are given as mean ±SD for six animals in each group

Parameters	Control	Diabetic	Diabetic + insulin	Diabetic + methionine	Methionine
Hemoglobin (g/dl)	11.2 ± 1.2	9.0 ± 0.4[a]*	11.6 ± 1.4[b]*	9.6 ± 1.0[a]*	11.2 ± 0.9
Methemoglobin (g/dl)	0.08 ± 0.015	0.08 ± 0.02	0.089 ± 0.02	0.084 ± 0.03	0.082 ± 0.01
Glutathione (mg/dl)	28.75 ± 1.7	22.9 ± 1.5[a]†	28.8 ± 1.2[b]†	28.9 ± 2.0[b]†	30.8 ± 1.8
Ascorbic acid (mg/dl)	1.67 ± 0.24	1.29 ± 0.34[a]‡	1.46 ± 0.3	1.38 ± 0.3	1.78 ± 0.2
Lipid peroxides (nmol/ml)	3.06 ± 0.35	4.12 ± 0.65[a]†	2.98 ± 0.5[b]†	3.09 ± 0.38[b]*	3.11 ± 0.5
Ceruloplasmin (mg/dl)	16.45 ± 4.2	23.7 ± 3.4[a]†	22.8 ± 3.5†	22.7 ± 4.8[a]†	17.8 ± 4.5
α-Tocopherol (mg/dl)	1.49 ± 0.14	2.58 ± 0.39[a]†	1.72 ± 0.28[a]†	2.62 ± 0.4[a]†	1.36 ± 0.16

[a] Significantly different compared to control.
[b] Significantly different compared to diabetic.
* $P < 0.01$; † $P < 0.001$; ‡ $P < 0.05$.

Table 2 Activity of lipid peroxidation in the erythrocytes of control and experimental animals. Values are expressed as p moles of MDA equivalents/mg Hb. (Values are mean ±SD for six animals in each group)

Particulars	Control	Diabetic	Diabetic + insulin	Diabetic + methionine	Methionine
Untreated	1.01 ± 0.42	2.75 ± 0.22[a]*	1.28 ± 0.18[b]*	2.28 ± 0.5[a]†	1.12 ± 0.5
Incubated for 60 minutes	1.20 ± 0.41	2.76 ± 0.49[a]*	1.46 ± 0.30[b]*	3.56 ± 0.45[a]*	1.38 ± 0.41
Treated with 3% H_2O_2	3.35 ± 0.17	4.85 ± 0.28[a]*	3.54 ± 0.17[b]*	4.72 ± 0.16[a]*	2.83 ± 0.5
Treated with 0.75% H_2O_2 + sodium azide	9.23 ± 0.16	9.42 ± 0.53	9.30 ± 0.2	9.34 ± 0.26	9.53 ± 0.7
Treated with ferrous sulphate	5.52 ± 0.34	7.46 ± 1.36[a]†	5.93 ± 0.29[b]‡	6.46 ± 0.32[a]‡	5.23 ± 0.3
% Maximal release	35.8 ± 2.1	53.2 ± 3.5[a]*	39.0 ± 4.8[b]*	52.8 ± 3.9[a]*	30.6 ± 4.5

[a] Significantly different compared to control
[b] Significantly different compared to diabetic.
* $P < 0.001$; † $P < 0.01$; ‡ $P < 0.05$

Table 3 Activity of lipid peroxidation in the erythrocyte membrane of control and experimental animals. Values are expressed as n moles MDA equivalents/mg protein. (Values are mean ±SD for six animals in each group)

Particulars	Control	Diabetic	Diabetic + insulin	Diabetic + methionine	Methionine
Untreated	0.23 ± 0.05	0.29 ± 0.01[a]*	0.24 ± 0.09	0.29 ± 0.1[a]*	0.21 ± 0.08
Incubated for 60 minutes	0.30 ± 0.05	0.38 ± 0.02[a]‡	0.30 ± 0.05[b]†	0.37 ± 0.02[a]‡	0.29 ± 0.05
Treated with 3% H_2O_2	1.16 ± 0.09	2.0 ± 0.08[a]*	1.32 ± 0.05[b]†	2.06 ± 0.18[a]*	1.21 ± 0.11
Treated with 0.75% H_2O_2 + NaN_3	5.40 ± 0.92	5.48 ± 0.14	4.28 ± 0.28	5.34 ± 0.1	5.58 ± 0.9
Treated with ferrous sulphate	2.47 ± 0.25	2.75 ± 0.08[a]†	2.44 ± 0.17[b]‡	2.82 ± 0.12[a]†	5.52 ± 0.3
% Maximal release	21.3 ± 3.2	36.5 ± 2.8[a]*	30.8 ± 5.7[a]†[b]†	38.5 ± 3.9[a]*	21.5 ± 4.5

[a] Significantly different compared to control
[b] Significantly different compared to diabetic.
* $P < 0.001$; † $P < 0.01$; ‡ $P < 0.05$

Table 4 Lipid levels in plasma of control and experimental animals. Values are expressed as mg/dl plasma. (Values are given as mean ±SD for six animals in each group)

Parameters	Control	Diabetic	Diabetic + insulin	Diabetic + methionine	Methionine
Total lipids	302.1 ± 20.8	550.8 ± 45.2[a]*	327.4 ± 34.5[b]*	535.7 ± 53.2[a]*	310.5 ± 29.5
Cholesterol	82.3 ± 7.3	143.2 ± 14.0[a]*	80.0 ± 8.4[b]*	137.2 ± 10.0[a]*	82.3 ± 7.8
Triacyl glycerol	80.3 ± 8.5	186.7 ± 14.6[a]*	86.2 ± 7.2[b]*	177.2 ± 10.6[a]*	84.2 ± 8.3
Phospholipids	92.8 ± 9.6	128.0 ± 12.1[a]*	98.2 ± 10.6[b]*	116.1 ± 8.8[a]*	94.3 ± 10.6

[a] Significantly different compared to control.
[b] Significantly different compared to diabetic.
* $P < 0.001$

normal value. However, methionine treatment has no effect. Oxidative stress has been shown to cause changes in the structure and function of hemoglobin,[25] resulting in hemoglobin desaturation and precipitation inside the red cells as Heinz bodies. It has been suggested that the physical presence of these Heinz bodies in the erythrocytes reduces the deformability of the cell, and binding of these inclusion bodies to the membrane results in osmotic damage with consequent lysis.[26] The increase in the mean corpuscular fragility (MCF) indicates the presence of increased proportion of fragile cells. The increase in osmotic fragility in diabetes may be attributed to factors such as altered membrane permeability and increase in volume/surface area ratio of erythrocytes. Decreased erythrocyte survival in alloxan diabetic rats.[27] and increased osmotic fragility of human erythrocytes in diabetes[28] have been reported. The deformability of erythrocytes is an important factor in the physiology of microcirculation. Reduced deformability of diabetic erythrocytes is suggested to contribute to circulatory dysfunctions.[29]

Elevated concentration of thiobarbituric acid-reactive substances (TBARS) in blood is noted in diabetic condition. Both administration of insulin or feeding methionine is found to restore its level to normal. The increase in TBA-reactivity in the plasma of diabetic rats may not merely be reflecting the increased plasma total lipid. This is supported by the fact that the plasma peroxide levels are restored to normalcy on treatment with methionine even though the plasma lipid levels are not restored. Increase in the level of lipid peroxides in blood generally is thought to be the consequence of increased production of and liberation into the circulation of tissue lipid peroxides due to pathological changes.[30] Sato et al.[31] have observed increased lipid peroxidation in the plasma of diabetics. Glavind et al.[32] have found high levels of lipid peroxides in the atheroma of diabetic patients.

The level of antioxidants regulate lipid peroxidation reactions in blood. Changes in the antioxidant levels are observed in the diabetic condition. Increase in α-tocopherol and ceruloplasmin and decrease in ascorbic acid and reduced glutathione levels have been noted.

Elevated α-tocopherol content observed in the plasma is compatible with the hypothesis that α-tocopherol excess in the plasma of diabetics plays a protective role against increased peroxidation and, hence, against increased platelet aggregability. Increase in α-tocopherol level may also be due to the increase in plasma total lipid levels. This is supported by the finding that when hyperlipemia is lowered by insulin treatment, α-tocopherol levels also decline. Increase in the ceruloplasmin levels is observed under conditions which involve generation of oxygen products such as superoxide radical and hydrogen peroxide.[33] Alteration in blood glutathione level is observed in diabetes when compared to control. Glutathione is known to protect cell structure by maintaining essential -SH groups of proteins and other molecules. It also participates in the destruction of hydrogen peroxide, other peroxides, and free radicals.[34] Alterations in glutathione levels have been reported in diabetes. Decreased total glutathione levels in erythrocytes of untreated diabetics[35] and in liver of streptozotocin-induced diabetic rats[36] have been reported. Intraperitoneal administration of insulin or oral methionine treatment have been found to restore glutathione level to normalcy. Dietary methionine provides a significant source of sulphur for the intracellular glutathione synthesis, thereby improving the thiol status of the cell. Treatment of diabetic rats with oral glutathione increases cytosolic superoxide dismutase (SOD) activity, an enzyme with antioxidant function in renal cortex and liver.[37]

Lipid peroxidation studies on the erythrocyte and erythrocyte membrane have shown higher release of TBARS in diabetic animals following exposure to hydrogen peroxide. Lipid peroxidation is found to be maximum in the presence of azide, and TBARS release, measured under this condition, represents the amount of polyunsaturated fatty acids present in the RBC membrane. The TBARS release without catalase inhibition is considered to be a reflection of the erythrocyte membrane antioxidant protection and the TBARS release with catalase inhibition as the maximal release possible.[22] The percent maximal release of TBARS is enhanced in the erythrocytes of diabetic rats. Insulin treatment has restored the erythrocyte lipid peroxidation to normal. However, methionine-treated rats showed elevated percent maximal release of TBARS in erythrocyte and erythrocyte membrane. This suggests that the membrane lipid composition is the same in both diabetic and methionine-treated condition. This is supported by the fact that the qualitative lipid composition of the membrane influences its susceptibility to peroxidation.[38] Furthermore, significant quantities of TBARS formation occur through the destruction of unsaturated fatty acids of phospholipids, leading to the depletion of phosphatidyl ethanolamine after peroxidant injury in the membrane.[39] In the present study, the administration of methionine is found to restore some of the antioxidant levels and also to reduce the activity of plasma lipid peroxidation in diabetic rats. However, the susceptibility of erythrocytes to lipid peroxidation is still found to be high. This suggests that either the plasma lipid peroxide levels are not determined by erythrocyte action or that the methionine treatment operates by removing lipid peroxide rather than by preventing its formation.

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