Beneficial Effects of Antioxidants on Oxidative Stress and Diabetes-Induced Experimental Nephropathy

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Summary

A study was undertaken to evaluate the effect of antioxidants such as β-carotene, vitamin C and vitamin E on oxidative stress and streptozotocin-induced diabetic nephropathy in rats. The rats were rendered diabetic by a single intraperitoneal injection of Streptozotocin (50 mg/kg), which was assessed by determining the blood glucose levels. Serum TBARS and plasma GSH levels were estimated to assess the oxidative stress. Furthermore, parameters like BUN, serum creatinine and urinary albumin were determined to assess the effect of antioxidants on diabetic nephropathy. Treatment with β-carotene (10 mg/kg/day i.p.), vitamin C (10 mg/kg/day i.p.) and vitamin E (40 mg/kg/day i.p.) showed significant ameliorative effects on oxidative stress and nephropathy in diabetic rats. Moreover, β-carotene was found to be more potent in the amelioration of oxidative stress and diabetic nephropathy when compared with vitamin C and vitamin E that may be due to its potent antioxidant property.

Key Words: β-carotene, vitamin C, vitamin E, oxidative stress, nephropathy

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Introduction

Diabetes is a global burden on healthcare resources and its morbidity and mortality is continuously increasing. Diabetes often leads to microvascular complications, macrovascular complications, cardiomyopathy, retinopathy, neuropathy, encephalopathy and nephropathy (1,2). Diabetic nephropathy is characterized by decreased glomerular filteration rate (GFR), excessive deposition of extracellular matrix proteins (3,4), thickening of peripheral glomerular basement membrane (5), glomerular hypertrophy, tubulointerstitial fibrosis (6), increased excretion of albumin (7) and decreased creatinine clearance (8). Diabetic nephropathy involves the complex mechanisms such as increased oxidative stress and consequent formation of advanced glycation end products (AGE) (9), activations of protein kinase C (PKC), c-Jun N-terminal kinase (JNK), p38 mitogen activated protein kinases (MAPKs), NADPH oxidase (10,11,12,13) and increased expression of growth factors (14). It is widely accepted that increased oxidative stress due to persistent imbalance between the production of highly reactive molecular species (chiefly oxygen and nitrogen) and antioxidant defenses is responsible for the development and progression of diabetic nephropathy (15,16). Moreover, lipid peroxidation has been found to be involved in the pathogenesis of diabetic nephropathy (17).

The antioxidants neutralize the reactive oxygen species (ROS) and thereby inhibit the progression of diabetes-induced nephropathy (18). The antioxidant bioflavanoids such as quercetin and *Curcuma aromatica* have been shown to attenuate the oxidative stress-induced renal dysfunction in diabetic rats (19). *Curcuma aromatica* and *Curcuma longa* have been shown to inhibit nephropathy in diabetic rats due to their antioxidant property (20). Red cabbage (*Brassica oleracea*) has been noted to activate the defence systems like superoxide dismutase, catalase and total antioxidant capacity in diabetic rats (21). Resveratrol, a powerful antioxidant, has been reported to attenuate oxidative stress induced renal dysfunction in diabetic rats (22). Taurine, a non-essential sulfur-containing amino acid possesses antioxidant property and it ameliorates streptozotocin (STZ)-induced diabetic nephropathy in rats (23). Ginger has been noted to activate antioxidant defence systems and reduce lipid peroxidation and nephropathy in diabetic rats (24). β-carotene is a powerful antioxidant found principally in plants and algae. β-carotene, a member of carotenoids, is considered to be a provitamin since it is converted to active vitamin A. β-carotene treatment has been demonstrated to reverse diabetes-induced decrease in antioxidant defense system (25,26). Vitamin C improves basal metabolic rate and lipid profile in diabetic rats because of its antioxidant property (27,28). Moreover, treatment with vitamin C or vitamin E reverses albuminuria, inhibits the release of glomerular transforming growth factor- β (TGF-β) and reduces the glomerular size in diabetes. Furthermore, supplementation of vitamin E improves the antioxidant defence system and insulin sensitivity in rats fed with high fructose diet. In addition, vitamin E attenuates the high plasma malondialdehyde and improves the antioxidant status in diabetic rats (29). However, the comparative effect of various antioxidants in diabetic nephropathy is not yet investigated. Therefore, the present study has been designed to investigate the comparative effect of antioxidants such as β-carotene, vitamin C and vitamin E on oxidative stress and diabetes-induced experimental nephropathy in rats.

Methods

Materials: Streptozotocin and β-carotene were purchased from Sigma Chemicals (St. Louis, MO, USA). Vitamin C and vitamin E were purchased from S.D. Fine Chemicals (Mumbai, India). Blood glucose kit was purchased from Vital Diagnostic Pvt. Ltd. (Mumbai, India). Serum creatinine kit was purchased from Agappe Diagnostic Pvt. Ltd. (Kerala, India). Blood urea nitrogen kit was purchased from Erba Diagnostic Pvt. Ltd. (Germany). Urinary albumin kit was purchased from Crest Biosystems Pvt. Ltd. (Goa, India). All other chemicals used in the study were of analytical grade and purchased from commercial suppliers.

Animals: Wistar albino rats (150-250 gm) bred in-house at I.S.F. College of Pharmacy, Moga, Punjab, India, were maintained in a temperature and humidity-controlled room with a 12-h light/dark cycle with free access to standard diet and water. All animals used in this study were mature and healthy and were not subjected to any form of treatment/medication. Guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India were followed and the in-house animal ethical committee approved all experimental procedures.

Streptozotocin-induced Experimental Diabetes: The rats were rendered diabetic by a single intra peritoneal injection of streptozotocin (STZ) (50 mg/kg) dissolved in 0.1 M citrate buffer (pH 4.5) (30). Blood samples were collected by retro-orbital puncture after 48 hours of the injection of STZ for the glucose estimation and the animals having blood glucose level more than 200 mg/dl (31) were selected for the study. The treatment with β-carotene, vitamin C and vitamin E were started from the day 1 of STZ injection and continued till the end of study.

Assessment of diabetes by estimating blood glucose: Blood glucose was estimated by glucose oxidase/peroxidase method using commercially available enzymatic kit obtained from Vital Diagnostic Private Ltd., Mumbai, India. In this method, 1000 µl working glucose reagent was added to 10 µl of serum (obtained from blood samples), 10 µl of standard glucose (100 mg/dl) and 10 µl of purified water to prepare test, standard and blank sample respectively. All the test tubes were incubated at room temperature for 30 min. To each test tube, 1000 µl of purified water was added. The absorbance of test and standard samples were measured against blank at 505 nm spectrophotometrically (Beckman DU 640B, Nyon, Switzerland). The concentration of glucose was calculated using the following formulae:

$$\text{Concentration of glucose (mg/dl)} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \times 100$$

Assessment of Oxidative stress: The oxidative stress was assessed by estimating serum thiobarbituric acid reactive substances (TBARS) and reduced GSH concentrations in plasma.

Estimation of serum TBARS concentration: The lipid peroxidation was assessed by measuring malondialdehyde (MDA) concentration, which was assessed by TBARS. In this method, 1000 µl of 20% trichloroacetic acid was added to 100 µl serum (obtained from blood samples) in a test tube to which 1000 µl of 1 % TBARS reagent (mixture of equal volume of 1%

TBA aqueous solution and glacial acetic acid) was added, mixed, and incubated at 100ºC for 30 min. After cooling on ice, samples were centrifuged at 1000 x g for 20 min. The absorbance was recorded spectrophotometrically at 532 nm against suitably prepared blank solution. A standard curve using 1,1,3,3-tetraethoxypropane was plotted to calculate the concentration of TBARS.

Estimation of reduced Glutathione (GSH) concentration: GSH was estimated using previously reported method (32). In this method, 0.02 ml of fresh or citrated blood sample was added to 9.0 ml of distilled water to which 1.0 ml of phosphate buffer (pH 8.0) was added. 3 ml of this solution was placed into each of two Beckman 1-cm cells, using one to adjust the absorbance to zero. To the other, 0.02 ml, 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) absorbance was determined at 420 nm after 30 min. Results were expressed as mmoles (SH)/l blood. The concentration of reduced glutathione was calculated using the following formulae:

$$Co = 36.8 \times \text{Absorbance}$$

Where, Co - Original concentration
A - Absorbance at 420 nm

Assessment of Diabetic Nephropathy:

Estimation of Blood Urea Nitrogen (BUN): Blood urea nitrogen was measured by Glutaryldehyde (GLDH)-Urease menthod using commercially available assay kit (Erba Diagnostic Pvt. Ltd., Germany). In this method, 1000 µL working reagent was added to 20 µL of serum (obtained from blood samples), 20 µL of standard (100 mg/dl) and 20 µL of purified water to prepare test, standard and blank samples respectively. To each test tube, 1000 µL of purified water was added. The samples were mixed and the optical density was measured at 340 nm. The concentration of BUN was measured using the following formulae:

$$\text{Concentration of BUN (mg/dl)} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \times \text{Concentration of standard}$$

Estimation of Serum Creatinine: Serum creatinine was measured by Modified Jaffe's method using commercially available kit (Agappe Diagnostic, India). In this method, 1000 µL working reagent was added to 100 µL of serum (obtained from blood samples), 100 µL of standard creatinine (100 mg/dl) and 100 µL of purified water to prepare test, standard and blank samples respectively. To each test tube, 1000 µL of purified water was added. The samples were mixed and the optical density (T_1) was read 60 seconds after the sample or standard addition at 492 nm. The second reading (T_2) was taken exactly after 60 seconds of first reading at 492 nm. The concentration of serum creatinine was measured using the following formulae:

$$\text{Concentration of Creatinine (mg/dl)} = \frac{(T_2 - T_1) \text{ of test}}{(T_2 - T_1) \text{ of standard}} \times 2$$

Estimation of Urinary albumin: Urinary albumin was measured by Bromocresol Green (BCG) method using commercially available assay kit (Crest Biosystems Pvt. Ltd., Goa, India). In this method, 1000 µL of BCG reagent was added to 10 µL of serum (obtained from blood samples), 10 µL of standard (100 mg/dl) and 10 µL of purified water to prepare test, standard and blank samples respectively. All the test tubes were incubated at room temperature for 30 min. To each test tube, 1000 µL of purified water was added. The absorbance of test and standard was measured against blank at 630 nm spectrophotometrically. The concentration of urinary albumin was measured using the following formulae:

$$\text{Concentration of Albumin (mg/dl)} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \times 4$$

Experimental Protocol: Five groups of rats were employed in the present study and each group comprised of six animals.

Group 1. Control Group: The non diabetic rats were included in this group and citrate buffer was administered. All parameters were assessed weekly from 0 day to 35 days.
Group 2. Diabetic Control Group: The animals were made diabetic by administration of STZ (50 mg/kg, i.p, once) in citrate buffer. All parameters were assessed weekly from 0 day to 35 days.
Group 3. β-carotene Treated Group: The treatment with β-carotene (10 mg/kg/day i.p.) was started in diabetic animals on the same day of administration of streptozotocin and continued till 35th day. All parameters were assessed weekly from 0 day to 35 days.
Group 4. Vitamin C Treated Group: The treatment with vitamin C (10 mg/kg/day *i.p.*) was started in diabetic animals on the same day of administration of streptozotocin and continued till 35th day. All parameters were assessed weekly from 0 day to 35 days.
Group 5. Vitamin E Treated Group: The treatment with vitamin E (40 mg/kg/day *i.p.*) was started in diabetic animals on the the same day of administration of streptozotocin and continued till 35th day. All parameters were assessed weekly from 0 day to 35 days.

Statistical Analysis: Statistical analysis was performed using SigmaStat2 statistical software. All the results are expressed in mean ± SEM. The data obtained from various groups were statistically analyzed using one way ANOVA followed by post hoc Tukey's multiple range test. The $p < 0.05$ was considered to be statistically significant.

Results

Effect of Antioxidants on blood glucose: The significant increase in blood glucose level was noted in diabetic control rats from 7th day to 35th day of protocol when compared with the control group rats. The marked increase in blood glucose level was noted in diabetic control rats at 21st, 28th and 35th day of protocol. However, treatment with various antioxidants such as β-carotene (10 mg/kg/day *i.p.*), vitamin C (10 mg/kg/day *i.p.*) and vitamin E (40 mg/kg/day *i.p.*) showed a significant reduction in blood glucose levels at 21st, 28th and 35th day of treatment when compared with the diabetic control rats. Moreover, β-carotene treatment markedly attenuated the streptozotocin-induced increase in blood glucose levels at 21st, 28th and 35th day of treatment when compared with vitamin C and vitamin E treatment groups (Table 1).

Table 1. Effect of Antioxidants on Blood Glucose

Days of treatment	Control group	Diabetic control group	β-carotene treated group	Vitamin C treated group	Vitamin E treated group
0 day	84.56±3.21	86.81±3.22	90.49±5.00	101.38±2.73	94.57±5.33
7 day	87.78±3.75	244.44±10.39[a]	265.76±24.50	287.23±21.31	230.60±22.08
14 day	84.08±2.88	266.16±14.33[a]	285.65±33.96	238.13±15.41	308.91±4.64
21 day	90.92±2.16	276.05±7.47[a]	121.15±1.98[bcd]	195.61±13.44[b]	227.13±17.98[b]
28 day	84.19±4.07	359.77±17.64[a]	92.15±1.87[bcd]	135.92±6.57[b]	137.02±10.32[b]
35 day	83.35±3.00	374.58±12.46[a]	86.34±1.55[bcd]	114.48±6.15[b]	113.01±2.07[b]

Values are reported in mg/dl and expressed as mean ± S.E.M. (n=6). a = $p<0.05$ vs Control group; b = $p<0.05$ vs Diabetic control group; c = $p<0.05$ vs Vitamin C treated group; d = $p<0.05$ vs Vitamin E treated group. Data are statistically analyzed by ANOVA followed by Tukey's multiple range test.

Effect of Antioxidants on serum TBARS: There was significant increase in TBARS level in diabetic control rats from 7th day to 35th day of protocol as compared to control group rats and marked increase in TBARS level was noted at 28th and 35th day of protocol. Treatment with β-carotene (10 mg/kg/day *i.p.*), vitamin C (10 mg/kg/day *i.p.*) and vitamin E (40 mg/kg/day *i.p.*) showed a significant reduction in diabetes induced increase in TBARS level at 21st, 28th and 35th day of treatment as compared to diabetic control rats. Moreover, β-carotene treatment showed significant decrease in TBARS level at 28th and 35th day of treatment when compared with vitamin C and vitamin E treatment groups (Table 2).

Table 2. Effect of Antioxidants on Serum TBARS

Days of treatment	Control group	Diabetic control group	β-carotene treated group	Vitamin C treated group	Vitamin E treated group
0 day	4.27±0.03	4.29±0.03	4.29±0.02	4.31±0.01	4.31±0.02
7 day	4.29±0.02	5.87±0.04[a]	5.87±0.04	5.85±0.03	5.86±0.04
21 day	4.28±0.04	6.02±0.02[a]	5.73±0.03	5.89±0.03	5.85±0.02
28 day	4.27±0.04	6.70±0.01[a]	5.32±0.04[b]	5.65±0.01[b]	5.56±0.01[b]
35 day	4.27±0.03	6.80±0.02[a]	4.54±0.05[bcd]	5.00±0.02[b]	4.93±0.15[b]

Values are reported in nmol/ml and expressed as mean ± S.E.M. a = $p<0.05$ vs Control group; b = $p<0.05$ vs Diabetic control group; c = $p<0.05$ vs Vitamin C treated group; d = $p<0.05$ vs Vitamin E treated group. Data are statistically analyzed by ANOVA followed by Tukey's multiple range test.

Effect of Antioxidants on reduced GSH: Significant reduction in GSH level was noted in diabetic control rats from 7th day to 35th day of protocol as compared to control group rats. Marked reduction in GSH level was observed at 28th and 35th day of protocol. However, treatment with β-carotene (10 mg/kg/day *i.p.*), vitamin C (10 mg/kg/day *i.p.*) and vitamin E (40 mg/kg/day *i.p.*) resulted in a significant increase in diabetes induced decrease in GSH levels at 21st, 28th and 35th day of treatment when compared with the diabetic control rats. β-carotene

treatment markedly prevented the decrease in GSH levels at 28th and 35th day of treatment when compared with vitamin C and vitamin E treatment groups (Table 3).

Table 3. Effect of Antioxidants on Reduced Glutathione

Days of treatment	Control group	Diabetic control group	β-carotene treated group	Vitamin C treated group	Vitamin E treated group
0 day	4.35±0.07	4.34±0.05	4.38±0.18	4.34±0.08	4.30±0.06
7 day	4.37±0.16	3.09±0.10[a]	3.14±0.12	3.18±0.10	3.17±0.04
21 day	4.34±0.04	1.09±0.10[a]	3.57±0.10[b]	3.41±0.10[b]	3.37±0.12[b]
28 day	4.32±0.20	0.92±0.07[a]	4.05±0.10[bcd]	3.50±0.16[b]	3.40±0.06[b]
35 day	4.35±0.06	0.80±0.06[a]	4.13±0.05[bcd]	3.77±0.06[b]	3.66±0.14[b]

Values are reported in mmol (SH)/l Blood and expressed as mean ± S.E.M. a = $p<0.05$ vs Control group; b = $p<0.05$ vs Diabetic control group; c = $p<0.05$ vs Vitamin C treated group; d = $p<0.05$ vs Vitamin E treated group. Data are statistically analyzed by ANOVA followed by Tukey's multiple range test.

Effect of Antioxidants on Nephropathy in Diabetic rats: Nephropathy was assessed by estimating blood urea nitrogen, serum creatinine and urinary albumin excretion.

Effect of Antioxidants on BUN: The significant increase in BUN level was noted in diabetic control group from 7th day to 35th day of protocol when compared with control group and marked increase in BUN level was noted in diabetic control rats at 21st, 28th and 35th day of protocol. However, treatment with various antioxidants such as β-carotene (10 mg/kg/day *i.p.*), vitamin C (10 mg/kg/day *i.p.*) and vitamin E (40 mg/kg/day *i.p.*) significantly decreased diabetes-induced increase in BUN at 21st, 28th and 35 day of treatment when compared with diabetic control rats. Moreover, β-carotene treatment markedly decreased high BUN level in diabetic rats at 28th and 35th day when compared with vitamin C or vitamin E (Table 4).

Table 4. Effect of Antioxidants on Blood Urea Nitrogen

Days of treatment	Control group	Diabetic control group	β-carotene treated group	Vitamin C treated group	Vitamin E treated group
0 day	19.85±0.36	19.89±0.37	19.93±0.31	19.93±0.30	19.96±0.26
7 day	20.27±0.31	21.77±0.34[a]	21.66±0.20	21.70±0.26	22.24±0.33
14 day	20.18±0.27	25.52±0.19[a]	25.74±0.62	26.21±0.38	26.04±0.32
21 day	19.68±0.18	29.86±0.52[a]	23.55±0.11[b]	25.31±0.34[b]	25.87±0.57[b]
28 day	19.75±0.13	31.99±0.27[a]	20.67±0.33[bcd]	23.48±0.33[b]	25.20±0.30[b]
35 day	20.03±0.13	32.83±0.35[a]	20.11±0.19[bcd]	22.69±0.35[b]	23.97±0.28[b]

Values are reported in mg/dl and expressed as mean ± S.E.M. a = $p<0.05$ vs Control group; b = $p<0.05$ vs Diabetic control group; c = $p<0.05$ vs Vitamin C treated group; d = $p<0.05$ vs Vitamin E treated group. Data are statistically analyzed by ANOVA followed by Tukey's multiple range test.

Effect of Antioxidants on Serum Creatinine: Diabetic control rats showed a significant increase in serum creatinine level from 7th day to 35th day of protocol when compared with control group rats. There was marked increase in serum creatinine level which was noted in diabetic control rats at 14th, 21st, 28th and 35th day of protocol. However, treatment with antioxidants such as β-carotene (10 mg/kg/day *i.p.*), vitamin C (10 mg/kg/day *i.p.*) and vitamin E (40 mg/kg/day *i.p.*) resulted in decreased high serum creatinine level at 14th, 21st, 28th and 35th day of treatment when compared with diabetic control rats. Moreover, β-carotene treatment markedly reduced high serum creatinine level in diabetic rats at 28th and 35th day of treatment when compared with vitamin C and vitamin E (Table 5).

Table 5. Effect of Antioxidants on Serum Creatinine

Days of treatment	Control group	Diabetic control group	β -carotene treated group	Vitamin C treated group	Vitamin E treated group
0 day	1.06±0.03	1.01±0.10	1.04±0.05	1.02±0.05	1.03±0.02
7 day	1.07±0.06	1.82±0.04[a]	1.86±0.03	1.84±0.06	1.81±0.04
14 day	1.07±0.09	2.49±0.03[a]	1.84±0.01[b]	1.84±0.04[b]	1.83±0.04[b]
21 day	0.90±0.04	3.32±0.15[a]	1.25±0.08[b]	1.45±0.12[b]	1.42±0.07[b]
28 day	1.25±0.08	4.15±0.06[a]	0.96±0.04[bcd]	1.22±0.03[b]	1.31±0.03[b]
35 day	1.02±0.04	4.84±0.07[a]	0.93±0.07[bcd]	1.21±0.03[b]	1.28±0.08[b]

Values are reported in mg/dl and expressed as mean ± S.E.M. a = $p<0.05$ vs Control group; b = $p<0.05$ vs Diabetic control group; c = $p<0.05$ vs Vitamin C treated group; d = $p<0.05$ vs Vitamin E treated group. Data are statistically analyzed by ANOVA followed by Tukey's multiple range test.

Effect of Antioxidants on Urinary Albumin Excretion: The significant increase in urinary albumin excretion was noted in diabetic control group from 7th day to 35th day of protocol when compared with control group. Marked increase in urinary albumin excretion level was noted in diabetic control rats at 21st, 28th and 35th days of protocol. Treatment with various antioxidants such as β-carotene (10 mg/kg/day *i.p.*), vitamin C (10 mg/kg/day *i.p.*) and vitamin E (40 mg/kg/day *i.p.*) significantly prevented diabetes-induced increase in urinary albumin excretion level at 21st, 28th and 35 day of treatment when compared with diabetic control rats. Moreover, β-carotene markedly prevented high urinary albumin excretion level in diabetic rats at 21st, 28th and 35th day of treatment when compared with vitamin C or vitamin E (Table 6).

Discussion

Diabetes mellitus was chemically induced in rats by administrating single dose of streptozotocin (STZ) (50 mg/kg *i.p.*) which produces cytotoxicity to β-cells of islets of langerhans in selective manner (33) by increasing the activity of xanthine oxidase and poly (ADP-ribose) polymerase (PARP), which consequently cause apoptotic and necrotic cell death in pancreatic β-cells of islets of langerhans (34). STZ was used in the present study because of its properties such as selective β-cell cytotoxic and minimal toxic to other organs as compared to alloxan (35). Moreover, the half-life ($t_{1/2}$) of single dose of STZ is about 15 min which is higher than the $t_{1/2}$ of alloxan (1.5 min) (35).

Table 6. Effect of Antioxidants on Urinary Albumin Excretion

Days of treatment	Control group	Diabetic control group	β-carotene treated group	Vitamin C treated group	Vitamin E treated group
0 day	3.63±0.04	3.60±0.09	3.57±0.07	3.58±0.06	3.62±0.05
7 day	3.48±0.09	10.25±0.18[a]	10.27±0.18	10.29±0.17	10.28±0.17
14 day	3.62±0.10	10.60±0.18[a]	10.43±0.24	10.52±0.22	10.58±0.21
21 day	3.58±0.08	11.11±0.12[a]	8.77±0.39[bcd]	9.70±0.52[b]	10.32±0.17[b]
28 day	3.54±0.06	11.63±0.16[a]	6.18±0.05[bcd]	7.40±0.18[b]	8.16±0.06[b]
35 day	3.55±0.05	12.24±0.05[a]	4.18±0.05[bcd]	5.20±0.05[b]	5.51±0.20[b]

Values are reported in mg/dl and expressed as mean ± S.E.M. a = $p<0.05$ vs Control group; b = $p<0.05$ vs Diabetic control group; c = $p<0.05$ vs Vitamin C treated group; d = $p<0.05$ vs Vitamin E treated group. Data are statistically analyzed by ANOVA followed by Tukey's multiple range test.

In the present study, the single dose of STZ (50 mg/kg *i.p.*) was observed to produce hyperglycemia after 48 hours of administration. It is well documented that hyperglycemia-induced oxidative stress in diabetes could be the major cause of development and progression of diabetic microvascular complications such as nephropathy (36,37).The decrease in reduced glutathione and increase in serum thiobarbituric acid reactive substances (TBARS) are taken as the markers of oxidative stress (32,38). It is well documented that hyperglycemia-induced increase in oxidative stress produces lipid peroxidation and consequent generation of malondialdehyde (MDA) (6,38). The decrease in reduced glutathione (GSH) level has been observed during reduced intracellular antioxidant defense system (39). Hence, these parameters have been used in the present study to assess the degree of oxidative stress. In the present study, the increase in serum TBARS, which is an index of lipid peroxidation was noted in diabetic rats. Moreover, reduced glutathione level was noted to be decreased in diabetic rats. These results suggest the development of oxidative stress in diabetic rats. In the present study, the administration of β-carotene, vitamin C and vitamin E significantly attenuated the STZ-induced rise in blood glucose level. The β-carotene markedly attenuated the STZ-induced increase in blood glucose level. This observation is consistent with a recent study in which β-carotene has been reported to ameliorate STZ-induced rise in blood glucose level (40). The treatment with β-carotene, vitamin C and vitamin E significantly attenuated the diabetes-induced increase in TBARS level and decrease in reduced glutathione level. The β-carotene markedly reduced the high TBARS level and increased the level of reduced glutathione in diabetic rats as compared to vitamin C and vitamin E, which may be due to its potent antioxidant property. β-carotene, apart from its potent antioxidant property, has inhibitory effect on the formation of advanced glycation end products (AGE) by inhibiting the Maillard reaction (41). Thus, it may be suggested that the reduction in blood glucose level with β-carotene, vitamin C and vitamin E may be due to their antioxidant properties and reduction in oxidative stress in β-cells of islets of langerhans. This contention is supported by the fact that quercetin, an antioxidant flavanoid, showed protection on pancreatic β-cells by decreasing the oxidative stress in STZ-induced diabetic rats (42). Moreover, the result obtained in the present study is further supported by the recent report in which rutin, an antioxidant bioflavanoid has been shown to scavenge free radicals, inhibit lipid peroxidation and protect β-cells of islets of langerhans, resulting in increased insulin secretion and decreased blood glucose levels (43). The increase in blood urea nitogen and

serum creatinine are reported to be an index of altered glomerular filteration rate (GFR) in diabetic nephropathy (44). Further, the decrease in creatinine clearance occurs in diabetic rats with nephropathy due to progressive renal damage and reduction in GFR (44). Moreover, the urinary albumin level has been reported to be increased in diabetic nephropathy and is considered to be a marker of glomerular injury (22). In the present study, blood urea nitrogen, serum creatinine and urinary albumin were noted to be increased in diabetic rats which suggest the development of diabetic nephropathy. It has been reported that decreased GFR has been associated with the formation of reactive oxygen species (ROS) (45). In the present study, β-carotene, vitamin C and vitamin E have attenuated diabetes-induced increase in blood urea nitrogen, serum creatinine and urinary albumin in diabetic rats, which may be due to their reduction in the formation of ROS. The activation of polyol, PKC, AGE and hexosamine pathway have been shown to play a key role in the pathogenesis of diabetic nephropathy (12,13,46). It has been reported that diabetes-induced oxidative stress involves activation of various signaling pathways such as polyol, PKC, AGE and hexosamine. Thus, the ameliorative effects of antioxidants such as β-carotene, vitamin C and vitamin E in diabetes-induced nephropathy may be due to their antioxidant properties and consequent inhibition of polyol, PKC, AGE and hexosamine pathways. The β-carotene was found to be more potent in preventing diabetic nephropathy than vitamin C and vitamin E that may be due to its potent antioxidant property.

In conclusion, the findings of the present study strongly suggest that oxidative stress plays a key role in the pathogenesis of diabetic nephropathy. The treatment with antioxidants such as β-carotene, vitamin C and vitamin E produced ameliorative effect on nephropathy in diabetes, which may be due to their antioxidant properties. β-carotene was found to be more potent in the amelioration of diabetic nephropathy when compared with vitamin C and vitamin E that may be due to its potent antioxidant property.

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