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The beneficial effect of combined administration of vitamins C and E on renal function and selected parameters of antioxidant system in diabetic rats fed zinc-deficient diet

Derai El-hadjela[1,2]*, Kechrid Zine[1] and Bouhafs Leila[2]

[1]Laboratory of Biochemistry and Microbiology application, Department of Biochemistry, Faculty of Sciences, Badji Mokhtar University, Annaba, Algeria
[2]Department of Molecular and Cellular Biology, Faculty of Natural Sciences, University of Jijel, 18000 Jijel, Algeria.

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The aim of this study was to examine the progression of kidney damage induced by zinc deficiency in diabetic rats and to evaluate the effect of combined treatment of vitamin E and vitamin C in renal injury by providing protection against deleterious action of zinc deficiency. Female diabetic albino Wistar rats were randomly assigned into five groups. The first group received a diet containing a 54 mg zinc/kg diet (adequate zinc, AZ), the second group received a diet containing 1 mg zinc/kg diet (zinc deficient group, ZD), and the three other groups received ZD diet and treated orally with vitamin E (500 mg/kg body wt) (ZD + Vit E), vitamin C (500 mg/kg body wt) (ZD + Vit C), and combined vitamins C and E (250 + 250 mg/kg body wt) (ZD+VitC+VitE), respectively. Body weight was recorded regularly (twice weekly). After four weeks of dietary manipulation, kidney zinc level, serum albumin and total protein concentration of ZD group were significantly lower than those of AZ group. Dietary zinc deficiency also increased proteinuria excretion, serum and urinary urea and uric acid levels, serum creatinine and kidney malondialdehyde concentration. In contrast, the catalase activity and reduced glutathione level in the kidney were reduced. In conclusion, vitamins E and C act as beneficial antioxidants protect renal function against the noticed oxidative stress due to zinc deficiency and experimental diabetes.

Key words: Experimental diabetes, zinc, vitamin E, vitamin C, oxidative stress, kidney damage.

INTRODUCTION

Zinc is one of the most important essential metals for human nutrition. It is important for cellular processes, like genetic expression, cell division, and growth. This trace element is crucial for the function of more than 300 enzymes (Salgueiro et al., 2000; Jansen et al., 2009) and plays an important role in insulin action, carbohydrate and protein metabolism (Chausmer, 1998). Zinc deficiency has been reported to impact pancreatic function (Banavara et al., 2011) and appears to decrease the ability of the pancreas to respond to glucose, eventually

*Corresponding author. E-mail: eh_derai@yahoo.fr.

Abbreviations: GSH, Reduced glutathione; **CAT,** catalase; **BSA,** bovine serum albumin; **AZ,** adequate zinc; **ZD,** zinc deficient group; **MDA,** malondialdehyde.

leading to islet cell damage (Chiara et al., 2007). Zinc status was proposed to play a key role in the onset and/or progression of diabetes (Miao et al., 2013), as supported by several examples in both man and rodent models (Taylor, 2005). According to the study of Faurea et al. (2007), several complications of diabetes may be related to increased intracellular oxidants and free radicals associated with decreases in intracellular zinc and zinc-dependent antioxidant enzymes. Therefore, abnormal zinc metabolism could play a role in the pathogenesis of diabetes mellitus, wich is accom-panied by sever oxidative stress as a result of an increase oxygen free radical production.

Increased oxidative stress in diabetes is postulated to promote the development of myocardial injury, neuro-pathy, retinopathy and nephropathy (Kowluru et al., 2007). Diabetic nephropathy is a leading cause of end stage renal disease. It is characterized functionally by proteinuria and albuminuria and pathologically by glome-rular hypertrophy, mesangial expansion and tubulointer-stitial fibrosis; these findings are closely related to the loss of renal function (Eun et al., 2007). Because this damage occurs as a result of increased reactive oxygen species production, extensive investigation has evalua-ted the ability of antioxidants like vitamin C and vitamin E to ameliorate complications of diabetes (Robert et al., 2010).

Vitamins C and E can be used as antioxidants separately or in combination. Both vitamins act synergisti-cally to decreased renal oxidative stress, and kidney damage, and increased renal hemodynamics. In addition, vitamins C and E improve vascular function and structure, and prevent progression of diabetic complica-tions. Thus, the aim of this study was to examine the combined effects of vitamins C and E on kidney damage due to zinc defficiency in diabetic rats. We measured markers of oxidative stress and antioxidant state, serum and urinary parameters of renal function.

MATERIALS AND METHODS

Chemicals

Alloxan, 5, 5′dithiobis-(2-nitrobenzoic acid (DTNB), vitamin E (α-tocopherol), and vitamin C were purchased from Sigma Chemical Co (St Louis, France). All other chemicals used in the experiment were of analytical grade.

Animals

Female albino (Wistar) rats of 10 weeks of age, weighing 200 -250 g, were obtained from Pasteur Institute (Algiers, Algeria). Animals were acclimated for one week under the same laboratory conditions of photoperiod (12 h light/12 h dark) with a relative humidity of 40% and room temperature of 22 ± 2°C. Standard rat food and deionized water were available *ad-libitum.*

Induction of diabetes and diet

Diabetes was induced with fresh alloxan monohydrate solution using a previously described method (Pathak et al., 2011). Alloxan was intraperitoneally administered at a dose of 150 mg/kg body weight dissolved in citrate buffer (0.01 M, pH 4.5). Blood glucose was measured seven days after induction of diabetes on samples taken from tail vein. The diabetic state was confirmed by a glucose-meter (ACCU-CHEK, Roche Diagnostics, Paris, France) when the glucose concentration exceeded 14 mmol/l.

The diet for rats consisted of (in g/kg diet): Cornstarch 326, sucrose 326, protein 168 (egg white solids), lipids 80 (corn oil), fiber 40 (cellulose), vitamin mix (sigma) and mineral mix 40. The latter was formulated to contain either adequate (54 mg/kg) or deficient (1.2 mg/kg) quantities of Zn, as determined by atomic absorption spectroscopy. The mineral mix supplied (in g /kg diet) calcium hydrogen orthophosphate 13; disodium hydrogen orthophosphate 7.4; calcium carbonate 8.2; potassium chloride 7.03; magnesium sulphate 4; ferrous sulphate 0.144; copper sulphate 0.023; potassium iodide 0.001, manganese sulphate0.180 and zinc carbonate 0.1. The low Zn diet contained no additional zinc carbonate.

Groups

The rats were randomly assigned into five groups of 8 animals each. The first group received a diet containing a 54 mg zinc/kg diet (adequate zinc, AZ) (Southon et al., 1988), the second group received a diet containing 1 mg zinc/kg diet (zinc deficient group, ZD) for 28 days. The third and the fourth groups received ZD diet and treated orally with vitamin E (500 mg/kg) (ZD + VE) (Demiralay et al., 2007) and vitamin C (500mg/kg) (ZD+VC) (Kaida et al, 2010) respectively. The fifth group received ZD diet and in combination vitamins C and E (250 + 250 mg/kg body weight) (ZD+VC+VE) (Yanardag et al., 2007). The experimental procedures were carried out according to the National Institute of Health Guide-lines for Animal Care and approved by the Ethics Committee of our Institution.

Urine collection

At the end of the experiment, the rats were placed in metabolic cages and 24-h urine was collected for the measurement of urinary protein, urea and uric acid.

Blood collection and preparation of tissue samples

At the end of the experimental period (28 days) after overnight fast, rats were decapitated and blood samples were transferred into ice cold centrifuge tubes. The serum was prepared by centrifugation, for 10 min at 3000 revolutions/min and utilized for total protein, albumin, creatinine, urea and uric acid assays. Absolute kidney weight was determined, one fragment of kidney was rapidly excised, weighed, freeze-clamped at -196°C, ground under liquid nitrogen and stored at -20°C for oxidative stress parameters analysis, the second fragment was washed with isotonic saline (9 g sodium chloride/l distilled water) and blotted to dry at 80°C for 16 h and zinc concentration was determined.

Measurement of biochemical parameters

In serum

Total protein and albumin level in serum were determined with commercial kits from Spinreact, Spain, refs; total protein- 100129 and albumin- 1001020. Serum creatinine, urea and uric acid concentration were also measured utilizing commercial kits (Spinreact, Spain, refs; creatinine-1001113, urea-1001333 and uric acid 1001032).

In urine

Urinary urea, uric acid concentration and proteinuria excretion were measured utilizing commercial kits (Spinreact, Spain, refs; urea-1001333, uric acid 1001032 and proteinury- 100129).

Kidney zinc analyses

Dried kidney was heated in silica crucibles at 480°C for 48 h and the ash taken up in hot hydrochloric acid (11.7 M) for Zn analyses. Kidney zinc concentration was determined by atomic absorption spectrophotometer (Pye Unicam SP 9000 Hitchin, UK) after twenty-fold dilution with doubly distilled water. The accuracy of zinc recovery was checked using standard reference materials; bovine liver and wheat flour. These standards were prepared and analysed in similar conditions to the test items to assess recovery. The recovery of zinc in the standard reference material exceeded 96%.

Antioxidant parameters estimations

Preparation of homogenates

About 0.5 g of kidney was homogenized in 2 ml of buffer solution of phosphate buffer saline 1:2 (w/v; 1g tissue 2ml TBS, pH=7.4). Homogenates were centrifuged at 10000xg for 15 min at 4°C, and the obtained supernatant was used for the determination of antioxidant enzyme activity.

Determination of malondialdehyde (MDA)

Kidney homogenates were prepared at 10% (w/v) in 0.1 mol/L Tris-HCl buffer, pH 7.4, and MDA steady-state level was determined. MDA was measured according to the method described by Sastre et al. (2000). Thiobarbituric acid 0.67% (w/v) was added to aliquots of the homogenate previously precipitated with 10% trichloroacetic acid (w/v). Then the mixture was centrifuged, and the supernatant was heated (100°C) for 15 min in a boiling water bath. After cooling, n-butanol was added to neutralize the mixture, and the absorbance was measured at 532 nm. The results were expressed as nmol of MDA/g tissue.

Estimation of reduced glutathione (GSH) level

The GSH content of kidney homogenates was measured by the method of Ellman (1959). 0.5 g of fresh kidney was homogenized in 3 volumes of 5% TCA using Dounce homogenizer. The samples were centrifuged at 2000 rpm for 15 min. The supernatant (50µl) was diluted in 10 ml phosphate buffer (0.1 *M*, *p*H 8). Consequently, 20 µl of DTNB 0.01 M was added to 3 ml of the dilution mixture. The measurement was performed at 412 nm against a control prepared in the same conditions using 5% TCA. The concentration is expressed in mmoles of GSH / g of kidney. They are deducted from a range of glutathione (GSH), which was prepared with the same conditions as dosage did.

Assay of catalase (CAT) activity

The activity of catalase was estimated according to the method of Clairborne (1985). Determination of CAT activity depends on changes in absorbance result from the decomposition of H_2O_2 by CAT. This change is measured at 240 nm every min for 2 min. Enzyme activity was expressed as unit per mg protein.

Protein determination

Protein concentration in the kidney homogenates was determined by Bradford method, using bovine serum albumin (BSA) as a standard (Bradford, 1976).

Statistical analysis

One-way analysis of variance (ANOVA) followed by post hoc Tukey-HSD test were used for data analysis. Results are presented as mean ± SEM. Values were considered statistically significant if $p<0.05$.

RESULTS

Blood glucose

Figure 1 shows that blood glucose ($p < 0.001$) values were higher in ZD group than in AZ group. Blood glucose ($p < 0.001$) values were significantly lower in ZD + VE and ZD + VC groups in comparison with ZD group. Combined vitamin E and vitamin C treatements significantly reduced blood glucose ($p < 0.001$) when we compared to ZD group and ($p < 0.05$) when compared with ZD + VE.

Body and kidney weights

Figure 2 shows that there was marked reduction in the body weight of diabetic animals fed low zinc diet compared to that of AZ group ($p < 0.05$). In addition, absolute kidney weight in ZD group was significantly increased ($p < 0.01$). Oral administration of vitamin E and C increased the body weight compared to ZD group. Moreover, vitamins treatment led to significant reduction in the kidney weight ($p < 0.001$and $p < 0.01$).

Kidney zinc

The concentration of zinc in kidney is shown in Figure 3 for all studied groups. The concentration was significantly lower in the ZD group than in the AZ group ($p < 0.001$) and significantly higher in ZD + VE ($p < 0.05$), ZD + VC ($p < 0.001$) groups compared to ZD group.

Combined vitamin E and vitamin C treatements significantly elevated kidney zinc status ($p < 0.001$) compared to ZD group and a significant rise in kidney zinc concentrations compared to ZD + VE ($p < 0.05$) and ZD + VC ($p < 0.01$) groups.

Blood biochemical values

Serum albumin, total protein, creatinine, urea and uric acid values are shown in Table 1. Serum albumin ($p < 0.05$) and total protein ($p < 0.01$) values were lower in ZD group than in AZ group. In contrast, creatinine ($p < 0.001$),

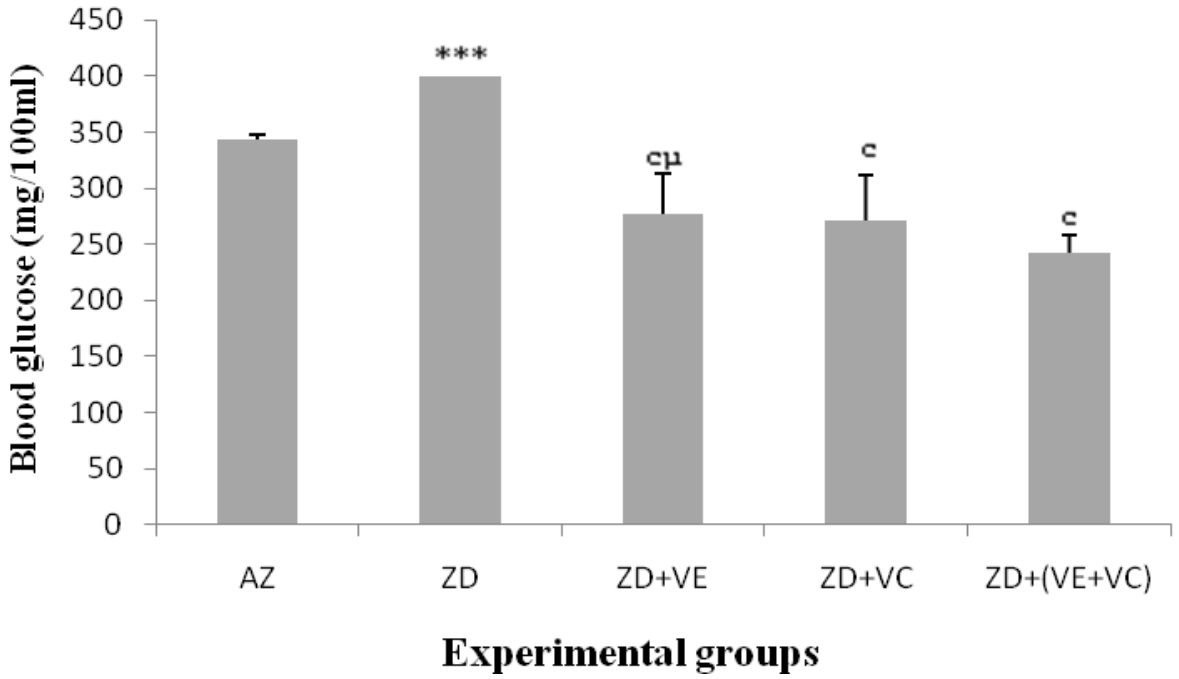

Figure 1. Blood glucose concentration in diabetic rats fed (AZ), (ZD) diets, (ZD+VE), (ZD+VC) and (ZD+VC+VE) after four weeks of treatment. Statistically significant differences from AZ: ***p < 0.001; from ZD: ^{c}p<0.001; from ZD+ (VE+V C): $^{\mu}$p<0.05. Values are given as mean ±SEM for group of 8 animals each.

Table 1. Serum albumin and creatinine levels, Urinary volume, Serum and urinary protein, urea and uric acid concentration of AZ, ZD, ZD+VE, ZD+VC and ZD+ (VE+VC) groups after four weeks of treatment.

Parameter	Experimental groups (n=8)				
	AZ	ZD	ZD+VE	ZD+VC	ZD+ (VE +VC)
Serum					
Total protein (g/l)	83.21± 2.6	76.38±0.59**	83.37±2.0^{c}	83.95 ±1.13^{c}	83.06 ±1.90^{c}
Albumin (g/l)	35.49±0.95	33.83±0.97*	35.46±0.15^{b}	35.82±0.52$^{c\mu}$	35.27 ±0.15^{b}
Urea (g/l)	0.57±0.04	1.02±0.13***	0.44 ±0.0 2^{ck}	0.47 ±0.04^{ck}	0.83±0.06^{a}
Creatinine (mg/l)	7.32±0.50	9.86±0.72***	7.29±0.17$^{c\mu}$	8.14 ±0.14^{bk}	7.58±0.14^{c}
Uric acid (mg/l)	32.00±6.08	57.33±0.57**	43.00±5.00^{b}	53.33 ±1.52^{ak}	36.33 ±2.51^{c}
Urine					
Urinary volume (ml/24 h)	33.25±6.18	50.50±11.35*	28.00 ±3.26^{b}	27.50 ±3.10^{b}	24.00 ±1.63^{b}
Protein (mg/24 h)	648.33±23.97	846.33±82.51*	437.9 ±17.3^{a}	601.33 ±28.92$^{b\mu}$	524.77±33.24^{b}
Urea (mg/24 h)	8.63±0.91	16.11±3.03**	9.23 ±1.82^{b}	10.83 ±0.79^{a}	10.60 ±0.51^{a}
Uric acid (µmol/24 h)	37.00±8.04	57.33±3.68**	38.33 ±11.26^{a}	28.30 ±23.40^{a}	42.15 ±10.77^{a}

Statistically significant differences from AZ: *P < 0.05, **P < 0.01, ***P < 0.001; from ZD: ^{a}P < 0.05, ^{b}P < 0.01, ^{c}P<0.001; from ZD+ (VC+VE): $^{\mu}$P<0.05, ^{K}P < 0.001. Values are given as mean ± SEM, n = 8 number of animals.

urea (p < 0.001) and uric acid (p < 0.01) values were higher than those of AZ rats. Serum albumin (p < 0.01 and p < 0.001) and total protein (p < 0.001) values were significantly higher in ZD + VE and ZD + VC groups in comparison with ZD group. Meanwhile creatinine (p < 0.001 and p < 0. 01), urea (p < 0.001) and uric acid (p < 0.01 and p < 0.05) values were lower in these two groups (ZD + VE and ZD + VC). Moreover combined vitamin E and vitamin C treatements significantly reduced creatinine (p < 0.001), urea (p < 0.05) and uric acid (p < 0.001) and elevated serum albumin (p < 0.01) and total protein (p < 0.001) values compared to ZD group, but administration of vitamin E and vitamin C in association improved creatinine (p < 0.05 and p < 0.001), urea (p <

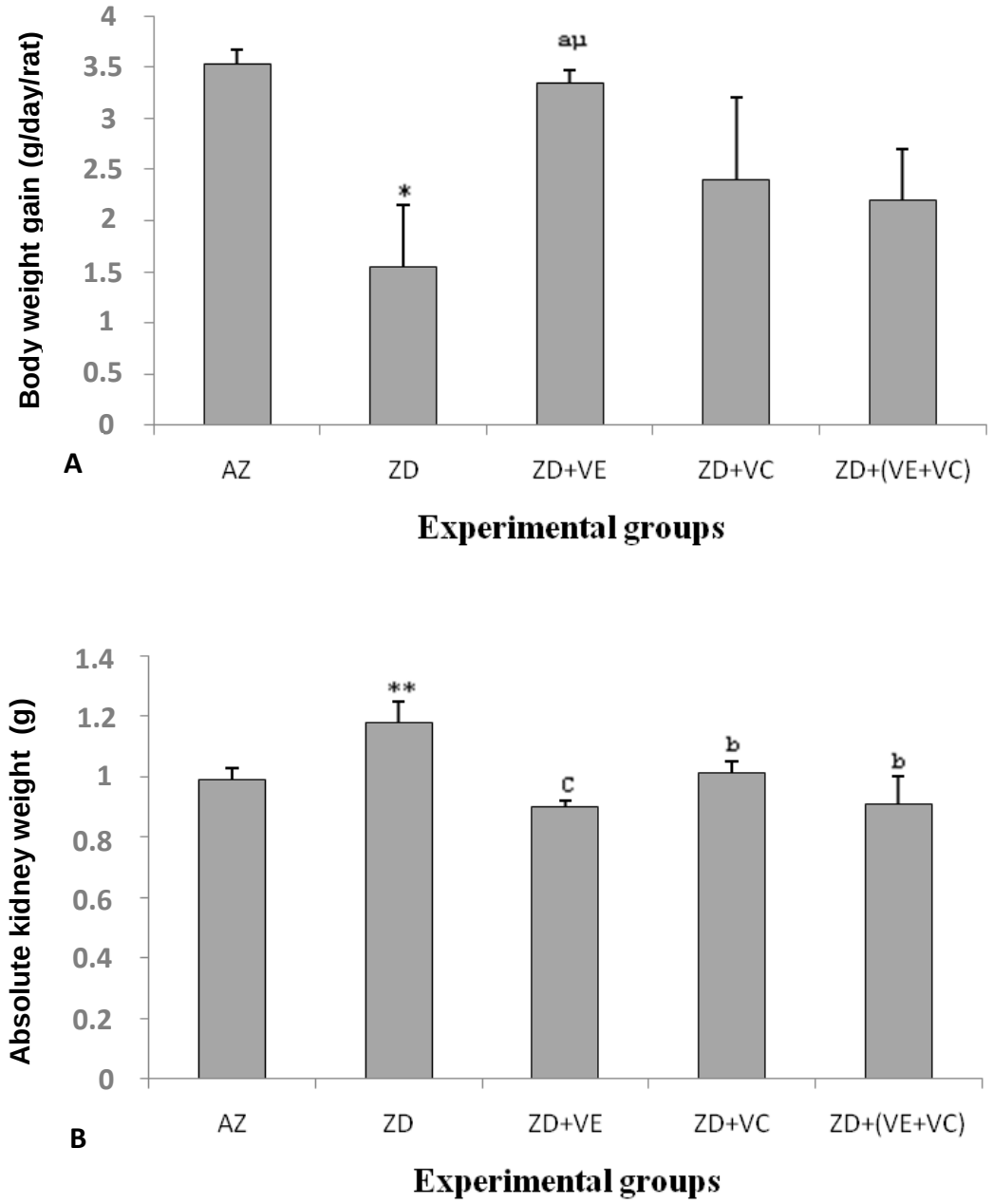

Figure 2. Body (A) and absolute kidney weight (B) of diabetic rats fed (AZ), (ZD) diets, (ZD+VE), (ZD+VC) and (ZD+VC+VE) after four weeks of treatment. Statistically significant differences from AZ: *$p < 0.05$, **$p < 0.01$; from ZD: $^{a}p < 0.05$, $^{b}P < 0.01$, $^{c}p<0.001$; from ZD+ (VE+V C): $^{\mu}P<0.05$. Values are given as mean ± SEM for group of 8 animals each.

0.001 and $p < 0.001$), uric acid ($p < 0.001$) and serum albumin ($p < 0.05$) values in comparison with (ZD+VE) or (ZD+VC).

Urinary biochemical values

Urinary protein, urea and uric acid values are shown in Table 1. Urinary protein ($p < 0.05$), urea ($p < 0.01$) and uric acid ($p < 0.01$) values were higher in ZD group than in AZ group. Urinary protein ($p < 0.05$ and $p < 0.01$), urea ($p < 0.01$ and $p < 0.05$) and uric acid ($p < 0.05$) values were lower in (ZD + VE and ZD + VC) groups respectively in comparison with ZD group. Moreover combined vitamin E and vitamin C treatements reduced, urea ($p < 0.05$) and uric acid ($p < 0.05$) concentrations compared to ZD group and improved urinary protein exerition ($p < 0.05$) in comparison with (ZD+VC).

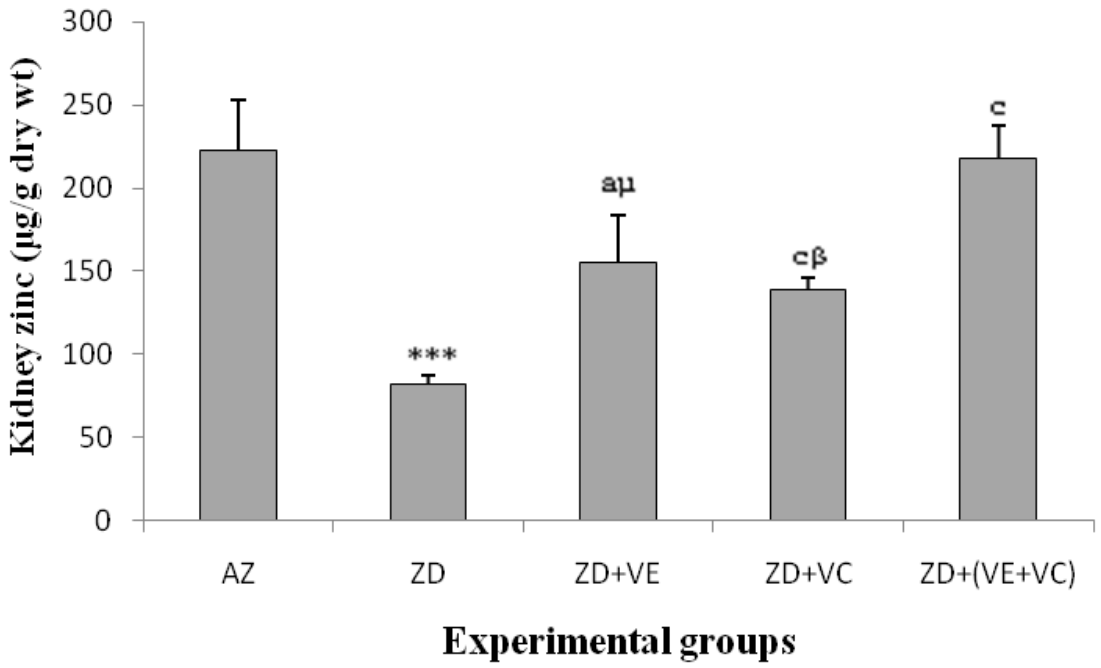

Figure 3. Kidney zinc level in diabetic rats fed (AZ), (ZD) diets, (ZD+VE), (ZD+VC) and ZD+(VC+VE) after four weeks of treatment. Statistically significant differences from AZ: ***$p < 0.001$; from ZD: $^{a}p < 0.05$, $^{c}p<0.001$; from ZD+ (VE+V C): $^{\mu}p<0.05$, $^{\beta}p < 0.01$. Values are given as mean ±SEM for group of 8 animals each.

Oxidative stress parameters

MDA concentration was significantly higher in diabetic animals fed low zinc diet as compared to the adequate zinc group ($P < 0.05$), but markedly declined after vitamin E and vitamin C administration ($P < 0.05$) compared to ZD group (Figure 4). Also, there was a marked decrease in the GSH level ($p < 0.01$), and CAT ($p < 0.01$) activity in ZD rats as compared to AZ group. There was a significant rise of GSH level ($p < 0.01$) and CAT activity ($p < 0.05$ and $p < 0.01$), in kidney of diabetic rats fed zinc deficient diet after vitamin E and vitamin C administration (Figure 4).

In addition administration of vitamin E and vitamin C in association reduced in part MDA and elevated GSH concentrations ($p < 0.01$), and CAT ($p < 0.05$) activity compared with ZD group, on the other part ameliorated CAT activity ($p < 0.05$) when compared with (ZD+VE) or (ZD+VC) groups.

DISCUSSION

The result of this study showed that there were marked reductions in the total body weight as well as elevation in the absolute kidney weight of rats fed the zinc deficient diet (ZD) compared with rats fed the adequate zinc diet (AZ). It was reported that growth retardation was common in zinc defficient bull calves this was due in part to decreased appetite and impaired protéin synthesis. Sun et al. (2006) reported that the relative weight of kidney increased by a zinc deficient diet and found that ZD diet influence organ development. Administration of vitamin C and /or vitamin E minimized body weight loss observed in diabetic rats fed low zinc diet and ameliorated both body and kidney weights.

Blood glucose was affected by low zinc diet. The observed higher blood glucose in the present study of low zinc animals may relate to altered glucose utilisation by tissues or to the increased rate of endogenous glucose production (Hendricks and Mahoney, 1972). The result of this study and those of previous studies show that vitamin E and vitamin C reduce blood glucose levels in diabetic animals. However, vitamin E as an antioxidant may help in the clearance of free radicals responsible for the complications of diabetes mellitus. In addition, it may also promote the absorption or uptake of glucose from the intestine and cells, respectively (Al Shamsi et al., 2004). Vitamin C was reported to help in improving plasma glucose in patients with type 2 diabetes (Afkhami and Shojaoddiny, 2007).

Several studies clearly demonstrated that hyperglycemia is an important causal factor in the development and progression of diabetic kidney disease (Haidara et al., 2009). The diabetic hyperglycemia induces elevation of serum levels of urea, uric acid and creatinine which are considered as significant markers of renal dysfunction (Shind and Goyal, 2003). Our results show significant

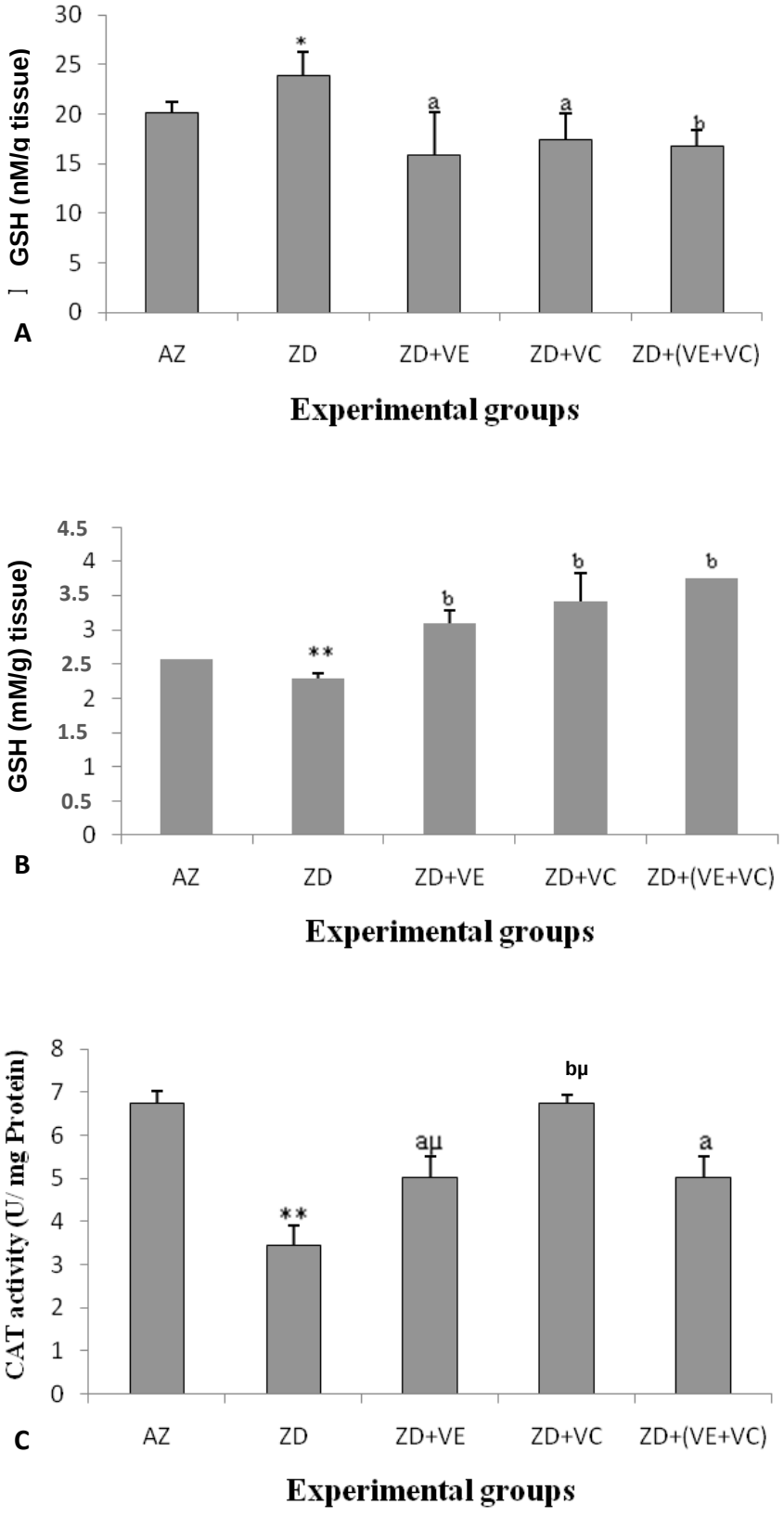

Figure 4. Kidney MDA (A) and GSH levels (B) and CAT activity (C) of AZ, ZD, ZD+VE, ZD+VC and ZD+ (VE+VC) groups after four weeks of treatment. Statistically significant differences from AZ: *p < 0.05, **p < 0.01; from ZD: ^{a}p < 0.05, ^{b}p < 0.01; from ZD+ (VE+V C): $^{\mu}$p<0.05. Values are given as mean ±SEM for group of 8 animals each.

increase in the level of serum urea, uric acid and creatinine in the diabetic rats fed low zinc diet. These results indicated that zinc deficiency elevated renal markers (serum urea nitrogen, uric acid and creatinine), which are found responsible for proper maintenance, functioning of kidney and change in the glomerular filtration rate (Mauer et al., 1981).

Our experimental data indicate that rats receiving a zinc-deficient diet had an increased rate of catabolism. This is based on the findings of elvated concentrations of urinary urea and uric acid. The production of uric acid, an end product of purine metabolism, can be influenced by altering the concentrations of substrates of nucleic acid. The urinary urea is the product of the hydrolytic cleavage of L-arginine by the enzyme arginase. The increased urinary urea in a zinc-deficient rat is due to the increase of hepatic arginase activity, which is a rate-limiting enzyme in urea formation (Jenc and william, 1975). Administration of vitamin E and C improves renal markers. Previous studies (Sutton et al., 1983; Mitch et al., 1981) suggest that vitamin C exerts a uricosuric effect by increasing urinary excretion and reducing serum concentrations of uric acid that at high levels could become crystallized in the joint and kidney and lead to gout and kidney stones.

Several studies have described biological mechanisms by which vitamin C reduces serum uric acid. *In vivo* studies suggest that vitamin C has uricosuric properties, increasing renal fractional clearance of uric acid, thereby reducing serum uric acid (Stein et al., 1976). This is likely due to competitive inhibition of an anion exchange transport system at the proximal tubule in the nephron (Berger et al., 1977). It is also possible that vitamin C increases the glomerular filtration rate by reducing glomerular microvascular ischemia and increasing dilatation of afferent arterioles (Huang et al., 2005).

In this experiment there was a significant reduction of kidney zinc, serum albumin and total protein concentration, but urinary protein excretion was elevated in zinc deficient group. A significant decrease in total protein level might be due to catabolism of protein and/ or malfunction of liver (Harper et al., 1977). In addition, changes in the binding of zinc to plasma proteins may result in an increase in ultrafilterable zinc concentration (Prasad and Oberleas, 1970). Administration of vitamins C and E decreased urinary protein and improves renal damage. These results are in agreement with the results of previous studies (Eun et al., 2007).

In the present study, GSH level and CAT activity were measured in renal tissue to evaluate the changes of antioxidant status in the kidney. Increased renal MDA content and decreased GSH level and CAT activity were found in diabetic rats fed low zinc diet compared to the AZ group. However, administration of vitamin E and C alone or in combination significantly improved these parameters. These vitamins may exert their beneficial effects through antioxidant action (Sadi et al., 2012). Vitamin C also acts as an aldose reductase inhibitor reducing sorbitol conversion and decreasing cellular damage in the kidney (Vincent, 1999). Vitamin E, on the other hand, acts as a non-enzymatic antioxidant and reduces lipid peroxidation and glutathione (Punithavatki et al., 2008; Minamiyama et al., 2008).

Conclusion

Our data shows that vitamin C or vitamin E alone and a combination of them preserved renal antioxidant levels and prevented kidney damage. The decreases in urinary protein excretion and the improvement in renal function in antioxidant vitamins treated groups suggest a major role of oxidative stress in the developing of renal dysfunction in diabetes associated with zinc deficiency.

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