Effects of manganese and vitamin E deficiencies on antioxidant enzymes in streptozotocin-diabetic rats

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Vitamin E and manganese deficiencies have been shown independently to affect the capacity to scavenge endogenously produced reactive oxygen species (ROS) in streptozotocin (STZ)-diabetic, Sprague-Dawley rats. Whether combined vitamin E and manganese deficiencies would additively affect oxidative stress was assessed in this study. Plasma and hepatic vitamin E were severely depleted in vitamin E-deficient rats, and susceptibility to lipid peroxidation in kidney, heart, liver, and pancreas tissues was increased, independent of manganese. Activities of key antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase in heart, liver, kidney, and pancreas were altered by manganese and vitamin E deficiencies, although no additive effects were observed. Hemoglobin glycosylation was elevated in vitamin E-deficient, diabetic rats, an effect that further underscores the increased oxidative stress in vitamin E deficiency states.

Keywords: manganese; vitamin E; antioxidants; diabetes; deficiency

Introduction

The importance of oxidative stress, resulting from decreased capacity to scavenge endogenously produced reactive oxygen species (ROS), increased production of ROS, or both, in contributing to the onset of secondary complications in diabetes, has been emphasized in a number of recent studies.[1-4] Inasmuch as levels of oxidative stress may be susceptible to dietary manipulation, the need for a better understanding of the interrelationships between antioxidant nutrients becomes clear.[5]

Manganese is an essential component of the mitochondrial oxidant scavenging enzyme, manganese superoxide dismutase.[6] Dietary deficiency of this micronutrient results in decreased activity of the enzyme and accompanying decreased resistance to lipid peroxidation in mitochondrial homogenates.[7] Manganese (Mn) deficiency has been shown to adversely affect glucose tolerance in mice, rats, guinea pigs, and chickens.[8-11]

Vitamin E is a micronutrient whose deficiency results in impaired resistance to oxidative stress.[12,13] Vitamin E deficiency enhanced the diabetogenicity of streptozotocin in rats, while vitamin E supplementation had the opposite effect.[14] In a follow-up study, vitamin E-deficient rats were susceptible to induction of diabetes at lower levels of streptozotocin (STZ), had lower levels of pancreatic manganese superoxide dismutase (MnSOD), and demonstrated an impaired response to glucose tolerance testing.[15] Selenium has been shown to additively enhance the increased susceptibility to lipid peroxidation of vitamin E deficiency.[16]

Recently, it was observed that manganese deficiency exacerbated the tissue antioxidant deficits of STZ-induced diabetes.[17] The current study was undertaken to determine if additional deficits would be 'unmasked' in the presence of a combined vitamin E and manganese deficiency.

Methods and materials

Chemicals and reagents

All chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO USA), unless otherwise specified.

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Diet components were obtained from ICN Biochemicals (Cleveland, OH USA).

Animals and diets

Weanling, male Sprague-Dawley rats (Charles River, Quebec, Canada) were individually housed in suspended, stainless-steel screen bottom cages in a temperature- and light-controlled room (294 +/− 1° K, 21° C, 12-hr light-dark cycle). Animals were fed ad libitum purified diets that were either Mn-deficient (1 ppm) or Mn-sufficient (45 ppm) and either vitamin E-deficient (3 ppm) or vitamin E-sufficient (44 ppm). Tocopherol-stripped lard replaced corn oil in all vitamin E-deficient diets. Composition of diets was the same as that of deRosa et al.[11] for Mn-deficient diets otherwise (30% protein, 54.5% carbohydrate, 8% fat, 6% salt mix, and 1.5% vitamin mix). Deionized water was provided ad libitum. Daily food consumption was recorded and rats were weighed weekly.

Induction of diabetes

All rats were initially maintained on purified diets for 8 weeks. Diabetes was induced by tail vein injection of STZ (60 mg/kg body weight for vitamin E-sufficient rats; 45 mg/kg body weight for vitamin E-deficient rats*). Diabetes was confirmed by blood glucose measurement (Accu-chek blood glucose monitor, Quebec, Canada) 24 hours after STZ treatment and again prior to autopsy. Blood glucose levels greater than 13 mmol/L were accepted as diabetic. All rats were fed purified diets for an additional 4 weeks following STZ injection.

Tissue collection

At termination, rats were anesthetized with halothane (MTC Pharmaceuticals, Cambridge, Ontario, Canada), and blood was obtained by cardiac puncture into heparinized syringes. Heart, liver, kidneys, and pancreas were removed, portions flash-frozen in liquid nitrogen, and stored at 203° K (−70° C) for ≤3 months pending measurement of reduced glutathione and analysis of enzymatic activity. The remainder was used for immediate lipid peroxidation assay.

Thiobarbituric acid reactive substances (TBARS) assay

Susceptibility to lipid peroxidation was assessed by measurement of thiobarbituric acid-reactive substances (TBARS), according to the method of Wohaieb and Godin.[18] Forced peroxidation was initiated by addition of *t*-butyl hydroperoxide (0.4 mmol/L) to red cell or tissue homogenates. Concentrations were calculated from a standard curve using $O.D._{532\,nm}$ of malondialdehyde bis(diethylacetal), 98% (Aldrich Chemical Co., Milwaukee, WI USA) as the standard.

Glycohemoglobin determination

Glycohemoglobin was determined colorimetrically as percent HbA_{1c} according to the method of Winterhalter.[19]

*For vitamin E-deficient rats, it was necessary to adjust the dosage of STZ down to 45 mg/kg body weight to achieve the same degree of hyperglycemia as vitamin E-sufficient groups without excessive mortality.[14]

Superoxide dismutase assays

For assay of MnSOD and CuZnSOD, a modification of the epinephrine autoxidation method[20] as outlined by Sun and Zigman[21] was employed. One unit is equivalent to the amount of enzyme required to inhibit the autooxidation by 50%.

Glutathione peroxidase and glutathione reductase assays

Glutathione peroxidase (GSHPx) was determined by an indirect, coupled assay procedure[22] as modified by Günzler and Flohé,[23] using *t*-butyl hydroperoxide as substrate. Glutathione reductase activity was measured as rate of disappearance of NADPH.[24]

Catalase assay

Catalase was determined according to the method of Aëbi[25] as modified by Davison et al.[26]

Vitamin E assays

Plasma samples were analysed spectrofluorometrically.[27] Plasma proteins were precipitated with absolute ethanol, vortexed, and the vitamin E extracted with hexane. The hexane and ethanol layers were then separated by centrifugation for 5 min at 2000 rpm. Fluorescence of the hexane (upper) layer measured spectrofluorometrically (Foci fluorometer with a xenon lamp; excitation wavelength 295 nm and emission wavelength 340 nm). DL-α-tocopherol, 2 mg/dL, was used as a standard for calculation of vitamin E concentrations in test samples.

Liver samples (approximately 200 mg/sample) were analyzed by high pressure liquid chromatography (HPLC).[28]

Protein determination

Tissue homogenate protein content was determined according to Lowry et al.[29]

Statistical analysis

All results, expressed as means +/− SEM, were compared using two-way analysis of variance initially to identify manganese and vitamin E deficiency effects and interactions. A one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests was used to identify within-group significant differences.[30]

Results

Food intake and weight gain were not affected by either Mn or vitamin E deficiency and were within normal ranges for diabetic rats.[18]

Plasma and hepatic vitamin E levels were severely depleted in vitamin E-deficient, diabetic rats, independent of manganese status (*Figure 1*).

Blood glucose levels did not differ among the four groups; however, glycosylated hemoglobin levels were elevated by vitamin E deficiency (*Figure 2*).

As expected, tissue susceptibility to H_2O_2-induced lipid peroxidation, as measured by tissue TBARS, was significantly increased in vitamin E-deficient, compared with vitamin E-sufficient, animals (*Table 1*). Manganese deficiency had no effect on susceptibility to H_2O_2-induced lipid peroxidation in tissue homogenates.

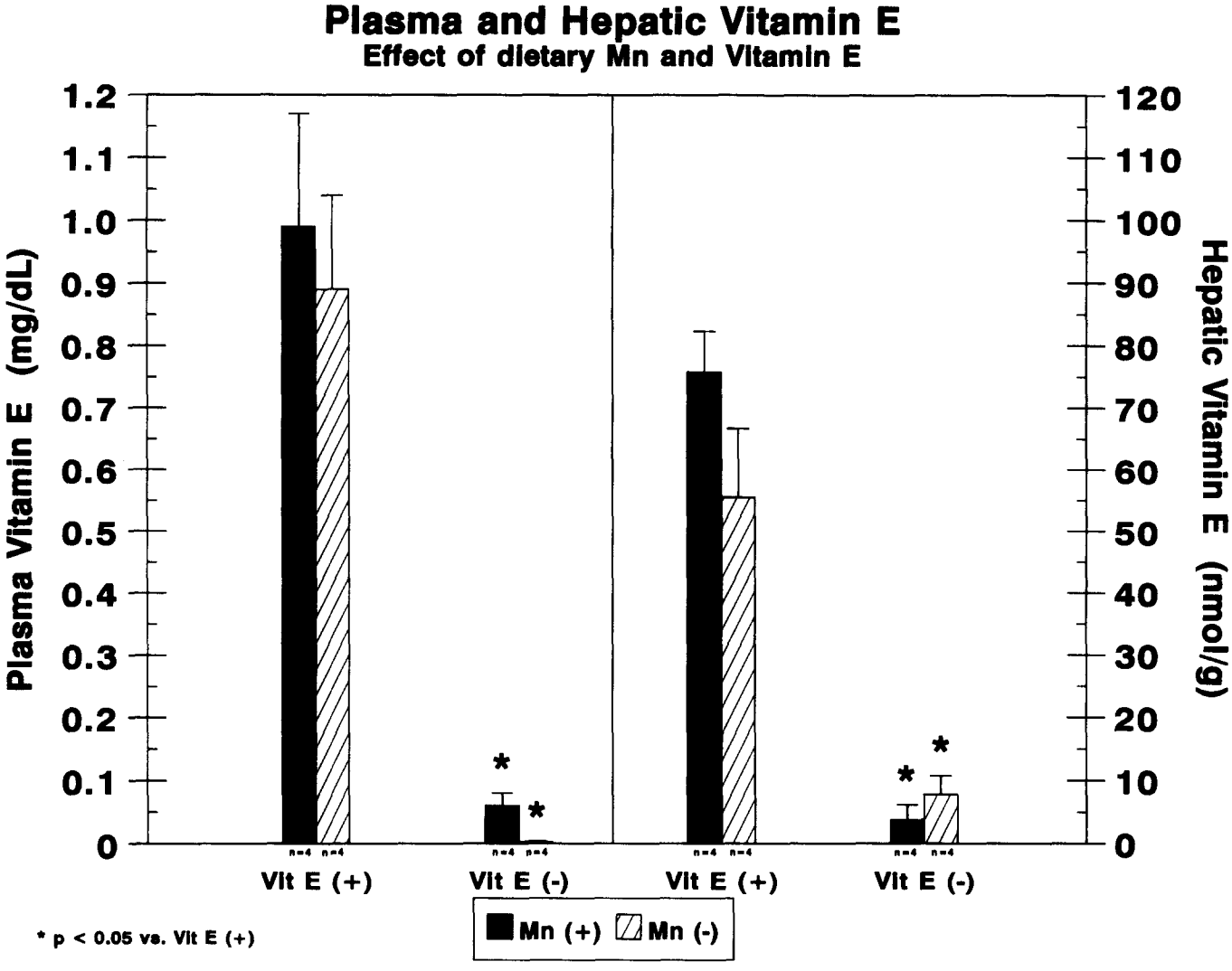

Figure 1 Plasma and hepatic vitamin E levels in manganese-sufficient and -deficient, vitamin E-sufficient and -deficient, diabetic rats. Data are means +/− SEM. The number of rats examined is reported within the brackets. Statistical analysis was performed using two-way ANOVA. Vitamin E-deficient versus vitamin E-sufficient = $P < 0.001$; manganese-deficient versus manganese-sufficient = NS.

Combined vitamin E and manganese deficiencies did not have any additive or synergistic effects on tissue antioxidant enzyme status. Mn deficiency depressed heart and kidney (but not pancreas) MnSOD activity, and increased liver CuZnSOD activity; however, there was no additive effect with vitamin E deficiency (*Table 2*). Pancreas MnSOD activity was decreased in vitamin E-deficient rats (*Table 2*). Vitamin E deficiency elevated heart GSHPx activity and diminished heart and liver GSSGRd activity (*Table 3*). Significant interaction terms for kidney MnSOD and for liver CuZnSOD (*Table 4*) are indicative of the fact that alterations in these enzymatic activities were significant only in vitamin E-sufficient animals.

Discussion

Rapid depletion of hepatic vitamin E in diabetic rats might be anticipated based on previous results showing depleted vitamin E in platelets of diabetic patients[31,32] and increased mortality in vitamin E-deficient rats treated with STZ at 55 mg/kg body weight.[14] Nonetheless, it is worth noting that the vitamin E depletion observed in this study with diabetic rats occurred within 12 weeks, while conventional vitamin E deficiency studies in rats require at least 6 months' feeding of the vitamin E-deficient diets.[13]

Vitamin E deficiency compromised pancreatic MnSOD activity and heart glutathione peroxidase activity, corroborating previous studies of antioxidant change in vitamin E-deficient (nondiabetic) rats;[13,15,33] however, an increased heart glutathione reductase activity was also noted. Heart and pancreas have been shown previously to be particularly susceptible to oxidative stress due to very low levels of key antioxidant enzymes, as compared with other tissues, such as liver and kidney.[34] The lack of any additive effects between vitamin E and manganese deficiency would suggest that there

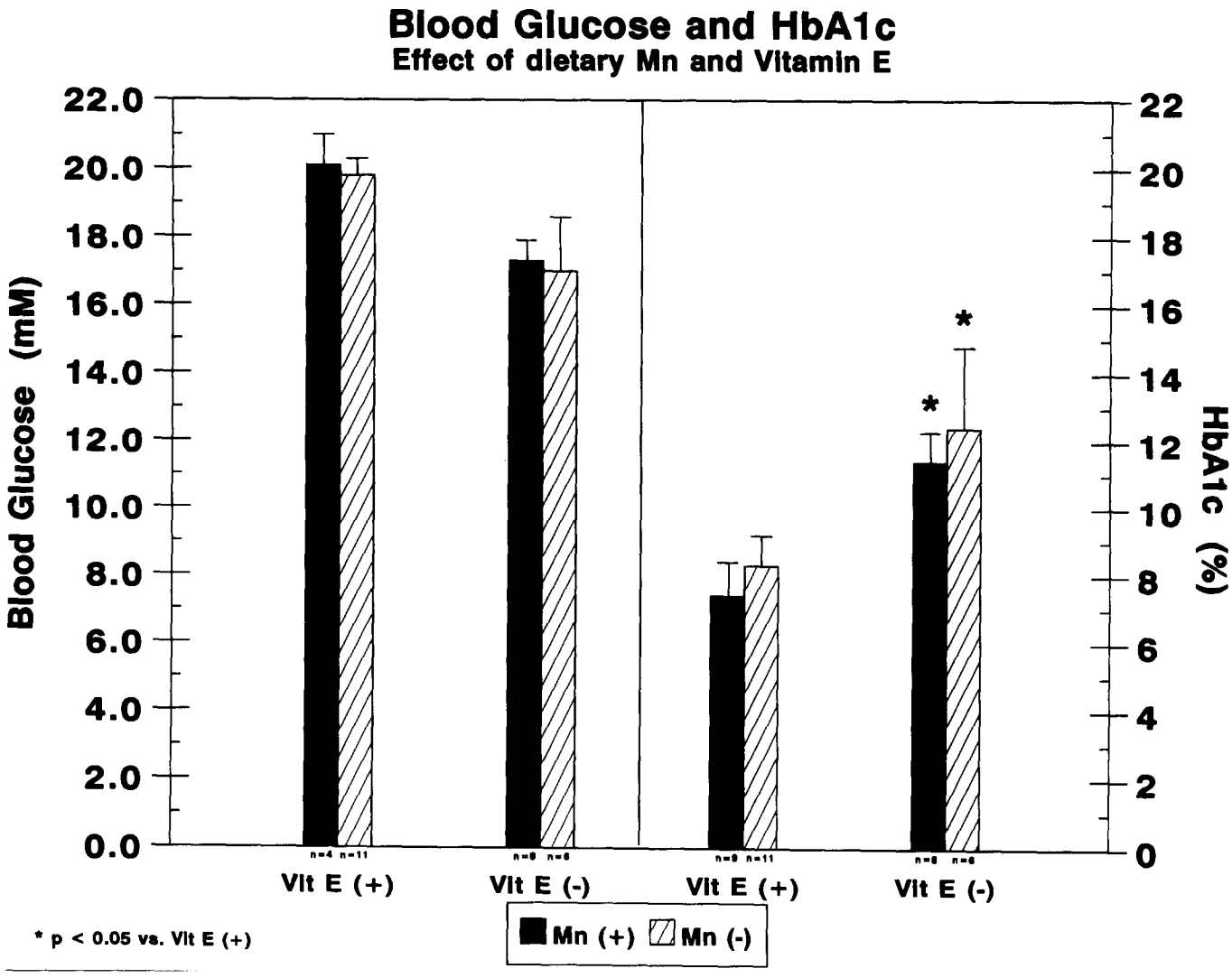

Figure 2 Blood glucose and HbA_{1c} levels in manganese-sufficient and -deficient, vitamin E-sufficient and -deficient, diabetic rats. Data are means +/− SEM. The number of rats examined is reported within the brackets. Statistical analysis was performed by two-way ANOVA. Vitamin E-deficient versus vitamin E-sufficient $\%HbA_{1c}$ = $P < 0.05$. Manganese-deficient versus manganese-sufficient = NS. There were no significant differences among blood glucose levels.

Table 1 Effects of vitamin E and manganese deficiencies on susceptibility towards lipid peroxidation in heart and kidney as determined by thiobarbituric acid reactive substances (TBARS)

	Vit E (+)		Vit E (−)	
	Mn(−)	Mn(+)	Mn(−)	Mn(+)
(n)	(11)	(9)	(6)	(8)
Heart TBARS	30.2[a]	36.2[a]	55.2[b]	67.3[b]
(nmol MDA/g tissue)	(± 2.4)	(± 3.1)	(± 7.0)	(± 7.4)
Kidney TBARS	44.3[a]	48.9[a]	67.7[b]	80.7[b]
(nmol MDA/g tissue)	(± 2.1)	(± 3.4)	(± 8.1)	(± 7.4)

Data are means ± SEM; the number of rats examined is reported within the brackets. Statistical analysis was performed using ANOVA. Means not sharing a common superscript were statistically different, $P < 0.05$.

may be other compensatory factors, such as ascorbic acid, uric acid, glutathione, etc., in protecting cells against increased production of ROS in diabetes mellitus.

Manganese-deficient, vitamin-E sufficient, diabetic rats had significantly lower levels of heart and kidney manganese superoxide dismutase and kidney glutathione peroxidase activities when compared with manganese-sufficient controls. These results confirm earlier studies in nondiabetic rats, which concluded that manganese deficiency by itself constitutes a state of increased oxidant stress.[35] The lack of a manganese effect on TBARS in this study is probably due to the use of whole tissue homogenates, rather than mitochondrial homogenates. In its role as a nutritive antioxidant, man-

Table 2 Effects of vitamin E and manganese deficiencies on tissue superoxide dismutase activities

	Vit E (+)		Vit E (−)	
	Mn(−)	Mn(+)	Mn(−)	Mn(+)
(n)	(11)	(9)	(6)	(8)
		MnSOD (U/g × 10^{-2})		
Heart	4.37[a] (± 0.23)	6.31[b] (± 0.41)	4.65[a] (± 0.21)	6.09[b] (± 0.30)
Kidney	3.92[a] (± 0.15)	5.47[b] (± 0.28)	4.98[ab] (± 0.30)	4.70[ab] (± 0.32)
Pancreas	3.09[a] (± 0.30)	2.88[a] (± 0.14)	2.36[b] (± 0.24)	2.35[b] (± 0.29)
		CuZnSOD (U/g × 10^{-2})		
Liver	104.2[a] (± 4.4)	84.1[b] (± 4.4)	98.5[ab] (± 3.8)	97.6[ab] (± 5.5)

Data are means ± SEM; the number of rats examined is reported within the brackets. Statistical analysis was performed using ANOVA. Means not sharing a common superscript were statistically different, $P < 0.05$. MnSOD, manganese superoxide dismutase; CuZnSOD, copper, zinc superoxide dismutase; U, units, where unit is amount required to effect 50% inhibition.

Table 3 Effects of vitamin E and manganese deficiencies on tissue glutathione peroxidase and glutathione reductase activities

	Vit E (+)		Vit E (−)	
	Mn(−)	Mn(+)	Mn(−)	Mn(+)
(n)	(11)	(9)	(6)	(8)
		GSSGRd (mol NADPH/min/g)		
Heart	0.103[a] (± 0.012)	0.127[a] (± 0.011)	0.192[b] (± 0.014)	0.210[b] (± 0.018)
Liver	2.34[a] (± 0.20)	2.33[a] (± 0.18)	2.95[b] (± 0.17)	3.04[b] (± 0.18)
		GSHPx (mol NADPH/min/g)		
Heart	8.35[a] (± 0.52)	7.87[a] (± 0.57)	6.96[b] (± 0.64)	6.72[b] (± 0.29)
Kidney	21.2[a] (± 1.1)	25.4[b] (± 2.7)	19.8[a] (± 1.2)	23.0[b] (± 1.4)

Data are means ± SEM; the number of rats examined is reported with the brackets. Statistical analysis was performed using ANOVA. Means not sharing a common superscript were statistically different, $P < 0.05$. GSSGRd, glutathione reductase; GSHPx, glutathione peroxidase.

ganese could be expected to improve metabolic function in other models of increased oxidative stress. In fact, manganese supplementation has been shown to ameliorate the compromised oxidative phosphorylation in ob/ob mice,[36] and to reduce the incidence of cataracts in oxidatively stressed rabbits.[37] Diabetes-induced alterations in tissue manganese levels have also been observed.[38–40]

An unexpected finding in this study was the increased hemoglobin glycosylation as a consequence of vitamin E deficiency in diabetic rats. This result corroborates earlier experimental evidence that glycosylation and peroxidative phenomena are interrelated.[3,41]

In conclusion, vitamin E and manganese deficiencies together did not additively increase oxidative stress in diabetic rats; nor did vitamin E deficiency 'unmask' any previously undisclosed interactive effects between manganese deficiency and STZ-induced diabetes. Nonetheless, antioxidant enzyme activities were adversely affected by both deficiencies. Vitamin E deficiency resulted in higher glycosylated hemoglobin levels at a lower dosage of STZ than vitamin E-sufficient animals; and plasma and vitamin E were severely depleted by 4 weeks following STZ treatment, indicating an increased potential for cellular damage due to unscavenged ROS in vitamin E-deficient rats, and suggesting a possible increased requirement for vitamin E in the diabetic state.

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Table 4 Effects of manganese and vitamin E deficiencies on tissue antioxidants in STZ-diabetic rats

Tissue	GSH	MnSOD	CuZnSOD	GSHPx	GSSGRd	CAT
Heart						
Mn	NS	<0.001	NS	NS	NS	NS
Vit E	NS	NS	NS	0.023	<0.001	NS
Interaction	NS	NS	NS	NS	NS	NS
Kidney						
Mn	NS	0.001	NS	0.025	NS	NS
Vit E	NS	NS	NS	NS	NS	0.023
Interaction	NS	0.028	NS	NS	NS	NS
Liver						
Mn	NS	0.016	0.017	NS	NS	NS
Vit E	NS	0.008	NS	NS	0.001	NS
Interaction	NS	NS	0.045	NS	NS	NS
Pancreas						
Mn	NS	NS	NS	NS	NS	NS
Vit E	0.004	0.021	NS	NS	NS	0.001
Interaction	NS	NS	NS	NS	NS	NS

GSH, reduced glutathione; MnSOD, manganese superoxide dismutase; CuZnSOD, copper, zinc superoxide dismutase; GSHPx, glutathione peroxidase; GSSGRd, glutathione reductase; CAT, catalase; NS, not significant. *P* values by ANOVA.

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