CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem Funct 2002; **20**: 75–80.
DOI: 10.1002/cbf.957

Effects of vitamin A and insulin on the antioxidative state of diabetic rat heart: a comparison study with combination treatment

Fulya Zobalı[1], Aslıhan Avcı[2], Orhan Canbolat[2], and Çimen Karasu*[1]

[1]*Department of Pharmacology, Faculty of Pharmacy, Ankara University, Ankara, Turkey*
[2]*Department of Biochemistry, Faculty of Medicine, Gazi University, Ankara, Turkey*

Because elevated oxidative stress may exacerbate cardiovascular complications of diabetes mellitus, the current study aimed to investigate the effects of treatment with either vitamin A, an antioxidant, or with insulin on lipid peroxidation products and antioxidant enzyme activities of diabetic rat heart. Also to evaluate whether a combination of vitamin A and insulin exerts more beneficial effects than treatment with each agent alone. Rats were made diabetic with a single injection of streptozotocin (STZ, 55 mg kg^{-1} i.p.). Two days after STZ-injection, one group of diabetic rats was treated with vitamin A (retinol acetate, 30 mg kg^{-1} day^{-1} i.o.) for 12 weeks. A second group of diabetic rats was untreated for 6 weeks and then treated for another 6 weeks with insulin (8–10 IU rat^{-1} day^{-1} s.c.). Both therapies were applied to another group of diabetic rats for assessment of combined therapy with vitamin A plus insulin. Hearts from 12-week untreated diabetic animals showed about a four-fold increase in the level of thiobarbituric acid reactive substances (TBARS), indicative of increased lipid peroxidation. This was accompanied by approximately 100% increase in both catalase and glutathione peroxidase (GSHPx) enzyme activities. Therapy with insulin alone caused a small but significant improvement in plasma TBARS as well as GSHPx activities, but no significant change in plasma catalase in diabetic animals. Diabetes-induced disturbance in TBARS was almost completely prevented by vitamin A therapy. Although, a similar degree of activities for GSHPx was determined in diabetic animals treated with each agent alone, combination therapy was found to be more effective than single therapies in the recovery of GSHPx of diabetic heart. In contrast to insulin single therapy, vitamin A alone significantly prevented an increase in catalase activity of diabetic heart, and a combination of these agents did not supply any further benefit. Superoxide dismutase (SOD) activity was not found significantly different among the experimental groups. STZ-diabetes also resulted in less plasma retinol and retinol-binding protein (RBP), which was significantly improved by insulin single therapy while vitamin A used alone, failed to increase plasma retinol and RBP levels of diabetic animals. Our findings suggest that single therapy with insulin is unable to preclude oxidative reactions in diabetic heart to the same extent as obtained by vitamin A therapy alone, in spite of allowing recovery of normal growth rate and improved vitamin A metabolism in diabetic rats. A combination of insulin with vitamin A may provide more benefits than use of either agent alone in the treatment of general characteristics of diabetes and the maintenance of antioxidant defence of diabetic heart and thus in the reduction of peroxidative stress-induced cardiac injury. Copyright © 2001 John Wiley & Sons, Ltd.

KEY WORDS — experimental diabetes; rat heart; vitamin A; insulin; oxidative stress; superoxide dismutase; catalase; glutathione peroxidase

INTRODUCTION

Diabetes mellitus, a chronic disease resulting from insufficiency of insulin secretion or action, is associated with cardiac complications such as ischaemic heart disease. Hyperglycaemia generates abnormally high levels of free radicals by autoxidation of glucose and protein glycation, and oxidative stress has been reported to be a causal factor of cardiovascular

* Correspondence to: Dr Ç. Karasu, Department of Pharmacology, Faculty of Pharmacy, Ankara University, 06100, Tandoğan, Ankara, Turkey. Tel: (90) 312 2126805 Ext.2226. Fax: (90) 312 2131080. E-mail: karasu@pharmacy.ankara.edu.tr
(Antioxidants in Diabetes-Induced Complications) The ADIC Study Group

Received 26 June 2001
Accepted 18 July 2001

complications in diabetes mellitus.[1] Diabetes also compromises natural antioxidant defence systems, changing antioxidant enzyme activities in tissues.[2–5] Previously it has been shown that antioxidants, including coenzyme Q10, probucol, and alpha-lipoic acid,[6–8] have some capacity to prevent or reverse disturbances in tissue antioxidant defence and cardiovascular abnormalities in diabetic animals.

Vitamin A is a lipid-soluble antioxidant that inhibits oxidation of biomolecules and regulates endogenous activities of scavenging enzymes in cigarette smoke-induced[9] or TCDD-induced oxidative stress.[10] In addition, insulin-dependent diabetes mellitus (IDDM) or its experimental model, STZ-induced diabetes, is associated with an impaired metabolic availability of vitamin A.[11] Abnormal metabolism of vitamin A has been described with decreased circulating levels along with decreased carrier protein, i.e. retinol-binding protein (RBP).[11,12] On the other hand, an insulin treatment regimen, although it results in recovery of normal growth rates and reversal of the typical symptoms and biochemical abnormalities of diabetes, has been shown to be unable to completely inhibit protein glycation, which can maintain increased oxidative stress in diabetic tissues.[13,14] Thus, we hypothesized that vitamin A may have a therapeutic role in the prevention of excess oxidative stress or abnormal antioxidant defence seen in STZ-induced diabetes, and the use of insulin and vitamin A in combination may provide further benefits. The object of this study was to investigate the effects of single therapies of either vitamin A or insulin on lipid peroxidation, and on endogenous antioxidant enzyme activities in diabetic rat heart and to evaluate whether treatments using a combination of vitamin A and insulin are more beneficial than use of either agent alone. To clarify vitamin A metabolism and its interaction with diabetes, we also measured plasma levels of retinol and RBP in experimental groups.

MATERIALS AND METHODS

Induction of diabetes and the treatment protocols

Diabetes was induced in male Wistar rats weighing 250–300 g after an overnight fast, by a single intraperitoneal (i.p.) injection of STZ freshly dissolved in saline at a dose of 55 mg kg^{-1} and verified 48 h later by measuring tail vein blood glucose; animals with a blood glucose of less than 300 mg dl^{-1} were excluded from the study.

Two days after injection of either STZ or vehicle, rats were divided into the following groups: (1) untreated-diabetic rats ($n = 10$); (2) diabetic rats treated with retinol acetate (30 mg kg^{-1} day^{-1}, orally) over 12 weeks ($n = 8$); (3) diabetic rats untreated for 6 weeks and then treated with insulin (8–10 IU day^{-1} per rat, s.c.) for 6 weeks ($n = 8$); (4) diabetic rats treated with both retinol acetate and insulin as in protocols 2 and 3 ($n = 8$); (5) control rats injected with vehicle alone ($n = 15$). The treatment dose of vitamin A was chosen according to a previous study, and retinol acetate (2 mg) was prepared in 0.1 ml corn oil as described previously.[15] Rats were maintained under standard housing conditions for 12 weeks before experiments were conducted. All animal procedures were in full accordance with 'The Principles of Laboratory Animal Care' (NIH publication No. 85-23, revised 1985).

Tissue and blood analysis

Blood glucose concentrations were measured with an Ames glucometer (Glucometer III, Bayer Diagnostics, France).

Thiobarbituric acid reactive substances (TBARS), as an index of oxidative stress were measured by a previously described method[16] in heart homogenates.

Superoxide dismutase (SOD) activity in heart homogenates was measured spectrophotometrically at 560 nm using the modified method of Spitz and Oberley.[17] The reaction mixture in 50 mM phosphate buffer (pH 7.8) consisted of SOD-induced inhibition of the reduction of nitro blue tetrazolium, using a free radical-generating system of 0.1 nM xanthine and an amount of xanthine oxidase to produce a rate of absorbance change of 0.025 U min^{-1}.

Catalase was measured spectrophotometrically by the method of Aebi.[18] Cleaned and minced hearts were homogenized in three volumes of 50 mM phosphate buffer (pH 7.0). The final volume of the mixture was made up to 2.0 ml by adding additional buffer solution. The reaction was started by the addition of 1.0 ml of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm. The enzyme activity for tissues was expressed as k mg^{-1} protein, where k is the first-order rate constant.

The method of Lawrence and Burk was used to measure glutathione peroxidase (GSH-Px) activity.[19] The assay mixture consisted of 75 mM phosphate buffer containing EDTA and NaN_3 (pH 7.0), 0.150 mg 10 000-g supernatant protein from the tissue, 0.1 mM NADPH, 4.0 mM GSH and 1.5 U glutathione reductase in a final volume of 500 µl. The reaction was started by the addition of 3.0 mM

H_2O_2. The rate of change of absorbance during the conversion of NADPH to $NADP^+$ was recorded spectrophotometrically at 340 nm for 3 min. GSH-Px activity was expressed as μmol of NADPH oxidized to $NADP^+$ min^{-1} mg^{-1} tissue protein.

Plasma retinol concentrations were determined by high-performance liquid chromatography as described previously.[20] For measuring serum retinol binding protein (RBP), a 'radial immunodiffusion assay' method was used in this study.[21]

Drugs and statistical analysis

All chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). Data are expressed as mean ± SEM. They were first subjected to Bartlett's test for homogeneity of variances and were log transformed if necessary. One-way analysis of variance was then performed, followed by the Student–Neuman–Keuls test to estimate the significance of differences for individual between-group comparisons.

RESULTS

All parameters assessed in this study are shown in Table 1. Diabetic rats lost weight and were hyperglycaemic. The combination of insulin with vitamin A exerted more beneficial effect on weight gain when compared with the single therapies. However, in the final analysis, the body weights of diabetic rats were still significantly lower than control rats. At the end of the treatments, insulin alone was found to be more effective than vitamin A alone in reducing blood glucose, and combination therapy showed an additive effect on blood glucose, but was unable to produce a normoglycaemia.

In untreated diabetic animals, TBARS was found to be four-fold higher than that in control rats. This was almost completely prevented by treatment with vitamin A while insulin alone was partially effective, and the effect of combined therapy on TBARS was not significantly different from the effect of vitamin A alone. SOD was found to be similar in the three experimental groups. GSHPx as well as catalase activity was significantly increased in untreated diabetic rats compared with the control group. Although single therapies with either vitamin A or insulin produced a similar degree of reduction in GSHPx activity of diabetic hearts, the combination of these treatment protocols led to more significant recovery in GSHPx activity when compared with the effects of single therapies. In diabetic hearts, although insulin treatment alone did not produce a significant reduction in catalase activity, vitamin A partially but significantly did so, however combined therapy was unable to produce any additional benefit.

Table 1. Body weights and levels of blood glucose, thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase, glutathione peroxidase (GSHPx), retinol and retinol binding protein (RBP) in animals

Parameters	Control $n=15$	Diabetic $n=10$	Insulin $n=8$	Vitamin A $n=8$	Insulin + Vitamin A $n=8$
Body weights (g)					
Start	272 ± 7	268 ± 10	276 ± 8	269 ± 6	271 ± 6
End	354 ± 10^{f}	153 ± 8^{c}	$237 \pm 8^{c,f}$	$189 \pm 5^{c,d}$	$261 \pm 11^{c,f}$
Blood glucose (mg dl^{-1})					
Start	100 ± 5	98 ± 3	102 ± 4	99 ± 5	108 ± 6
End	99 ± 3^{f}	464 ± 12^{c}	$213 \pm 10^{c,f}$	$382 \pm 8^{c,e}$	$175 \pm 7^{b,f}$
Heart TBARS (nmol mg^{-1} protein)	0.13 ± 0.003^{f}	0.45 ± 0.07^{c}	$0.35 \pm 0.04^{c,d}$	0.17 ± 0.02^{f}	0.19 ± 0.03^{f}
Heart SOD (units mg^{-1} protein)	0.93 ± 0.06	1.02 ± 0.05	1.04 ± 0.06	0.94 ± 0.04	1.18 ± 0.09
Heart Catalase (ks^{-1} mg^{-1} protein)	19.8 ± 0.53^{f}	38.7 ± 1.70^{c}	35.3 ± 3.16^{c}	$25.3 \pm 0.71^{a,f}$	$26.1 \pm 1.29^{a,f}$
Heart GSHPx (μmol min^{-1} mg^{-1} protein)	28.4 ± 2.1^{f}	60.1 ± 5.5^{c}	$43.4 \pm 4.3^{a,e}$	$41.7 \pm 6.0^{a,e}$	31.4 ± 4.0^{f}
Plasma retinol (ng ml^{-1})	294 ± 12^{f}	239 ± 9^{c}	287 ± 15^{e}	241 ± 6^{c}	288 ± 12^{e}
Serum RBP (mg dl^{-1})	6.79 ± 0.24^{f}	2.74 ± 0.12^{c}	$3.74 \pm 0.20^{c,e}$	3.07 ± 0.2^{c}	$3.86 \pm 0.18^{c,f}$

Control, untreated control rats; diabetic, 12-week untreated diabetic rats; Insulin, 6-week untreated and further 6-week insulin-treated diabetic rats; Vitamin A, 12-week vitamin A treated diabetic rats; Insulin + Vitamin A, 12-week diabetic rats treated with insulin plus vitamin A. Data are means ± SEM.
$^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus control, $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ versus diabetic.

Plasma retinol levels were weakly, but significantly, decreased in untreated diabetic rats compared with control animals. Insulin treatment, either alone or in combination with vitamin A, produced a significant increase in plasma retinol levels of diabetic animals. Serum RBP levels were dramatically decreased in untreated diabetic rats compared with controls. Although vitamin A alone failed to exert a significant effect on serum RBP levels of diabetic rats, the deficit in RBP was significantly but not completely reversed by treatment with insulin. There was no evidence for an additive effect of the two treatments on retinol and RBP.

DISCUSSION

Cardiovascular complications of diabetes mellitus commonly lead to considerable morbidity and mortality.[1] Increased oxidative stress due to increased oxygen free radical production is an important mechanism proposed to explain why poor glycaemic control of diabetes results in cardiovascular disease.[7,22] The increased production of reactive oxygen species is often associated with compromised natural antioxidant defence systems in diabetic tissues.[2–5] In response to oxidative stress, antioxidant enzymes are believed to be induced to protect cellular functions which maintain *in vivo* homeostasis.[2–5]

The present study has demonstrated that increased oxidative stress, as judged by elevated TBARS levels, is concomitant with decreased plasma retinol and RBP levels as well as increased heart GSHPx and catalase activities in an insulin-dependent experimental model of diabetes. These results are consistent with earlier reports, showing enhanced cardiac activities of GSHPx and catalase in diabetic rats,[5,6,23] and support the notion that when oxygen radical formation is sufficiently great, the oxidative stress can result in a compensatory increase in tissue activities of the free radical detoxifying enzymes.[24]

In the present study, the increases in cardiac GSHPx and catalase activities were observed in the absence of any significant changes in SOD, clearly indicating that diabetic heart is exposed chronically to peroxidative stress, due to elevated production of H_2O_2 *in vivo*. In accordance with the present findings, the results of our previous studies showed that the production and the biological availability of H_2O_2 are increased in diabetic vessels.[25,26] It is well known that H_2O_2 is normally detoxified in cells by either catalase and/or GSHPx, and the selective increase in H_2O_2 detoxifying enzymes but not SOD, has previously been shown in heart[3] and vessels[24] of diabetic animals. In diabetic heart, *in vivo* insulin treatment partially inhibited lipid peroxidation (TBARS), partially improved GSHPx, but did not significantly change catalase. Accordingly, it has been demonstrated that insulin treatment (6–12 U day^{-1}) attenuates or restores diabetes-induced changes in antioxidant enzymes in brain,[27] sciatic nerve,[28] some lymphoid organs[29] and kidney and heart.[2] Furthermore, in rats with STZ-induced diabetes, renal catalase mRNA levels were found to be greater than in normal rats, which were normalized by *in vivo* insulin treatment (6–10 U day^{-1}).[30] In this study, the effects of single therapy with insulin or vitamin A strongly suggested that the improvements observed in lipid peroxidation and antioxidant enzyme activities of diabetic hearts are unlikely to be linked totally with metabolic recovery. Although, insulin treatment alone, even started after 6 weeks of diabetes was induced, produced better metabolic control, as evidenced by improved growth and plasma glucose, retinol and RBP levels, than that obtained with vitamin A single therapy, it did not however, completely reverse the abnormalities in oxidative metabolism of diabetic heart. In contrast, vitamin A treatment alone, starting 2 days after diabetes induction, produced less beneficial effects on body weight and blood glucose than that obtained by insulin alone and failed to exert a significant effect on retinol and RBP levels. However, it almost completely prevented lipid peroxidation, and strikingly achieved the prevention of abnormalities in GSHPx and catalase activity in diabetic hearts. Our findings are consistent with previous studies showing that under diabetic conditions, the overall glycaemic control afforded by insulin treatment is not stringent enough to prevent excessive protein glycation, which can increase tissue oxidative stress.[13,14] It is clear that in diabetic tissue, the effects of different agents on oxidative stress depends largely on how they alter the balance between production and destruction of reactive oxygen species.

Our results emphasize once again the effectiveness of antioxidant therapy in the prevention of oxidative stress and hence peroxidative myocardial damage in diabetes. Vitamin A, is a member of the lipid-soluble retinoid compounds, plays a central role in the maintenance of normal cardiovascular functions[31] and can function as a lipoperoxyl-radical scavenger.[32] IDDM patients and STZ-induced diabetic rats have been shown to have impaired metabolic availability of vitamin A.[9,10,33] Chronic vitamin A deficiency and poorly controlled IDDM share some common clinical consequences such as increased risk of ischaemic heart disease.[33] Diabetes-induced changes in vitamin A metabolism, as indicated by its decreased circulatory

levels along with decreased carrier protein, RBP, is dependent on insulin deficiency. As supported by our findings, a lack of response to vitamin A supplementation has been shown to revert to normal by *in vivo* insulin treatment.[9,19,33] Our findings have also demonstrated that combination treatment with insulin plus vitamin A supplies a further benefit to the normalization of GSHPx activity and provides better body weight control and glucose metabolism in diabetic rats. Accordingly, it has been demonstrated that the serum levels of vitamin A are directly or indirectly correlated with SOD, GSHPx, catalase and MDA levels in blood.[34] Our study showed that in diabetic animals, a mild reduction in plasma retinol is not paralleled by a strong decrease in serum RBP, and that despite a weak restoration of serum RBP by insulin treatment, plasma retinol levels were almost completely reversed by insulin alone, suggesting that the dose regimen of insulin in our study may be insufficient to obtain complete improvement in the metabolism of vitamin A as well as of glucose.

In comparison with vitamin A single therapy, insulin treatment alone provided better control of glucose metabolism and improved weight gain, and partly improved vitamin A metabolism, but was unable to preclude increased oxidative reactions in diabetic rats however. Under the insulin treatment, diabetic animals still suffered an oxidative insult. In this study, we have shown for the first time that vitamin A single therapy effectively prevents oxidative stress by effects on endogenous antioxidant enzyme activities, but produces negligible changes in glucose and vitamin A metabolism in diabetic rats. However, when the GSHPx normalizing effect of combination therapy, and the separate effects of each agent on oxidative stress, antioxidant status, vitamin A and glucose metabolism are considered, the use of these agents in combination appears to have potential advantages especially in the reduction of peroxidative stress in diabetic conditions. The profound actions of two agents may protect cardiac functions against the detrimental effects of diabetes-induced peroxidative stress.

REFERENCES

1. Stemler J, Vaccaro O, Neaton D, Wentworth D. For the Multiple Risk Factor Intervention Trial Research Group. Diabetes, other risk factors and 12yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care* 1993; **16**: 434–444.
2. Asayama K, Hayashibe H, Dobashi K, Niitsu T, Miyao A, Kato K. Antioxidant enzyme status and lipid peroxidation in various tissues of diabetic and starved rats. *Diabetes Res* 1989; **12**: 85–91.
3. Wohaieb SA, Godin DV. Alterations in free-radical tissue-defense mechanisms in streptozotocin-induced diabetes in rat. Effects of insulin treatment. *Diabetes* 1987; **36**: 1014–1018.
4. Toleikis PM, Godin DV. Alteration of antioxidant status in diabetic rats by chronic exposure to physiological stressors. *Pharmaco Biochem Behav* 1995; **52**: 355–366.
5. Kakkar R, Mantha SV, Kalra J, Prasad K. Time course study of oxidative stress in aorta and heart of diabetic rat. *Clin Sci* 1996; **91**: 441–448.
6. Rauscher FM, Sanders RA, Watkins JB. Effects of coenzyme Q10 treatment on antioxidant pathways in normal and streptozotocin-induced diabetic rats. *J Biochem Mol Toxicol* 2001; **15**: 41–46.
7. Kaul N, Siveski-Iliskovic N, Hill M, Khaper N, Seneviratne C, Singal PK. Probucol treatment reverses antioxidant and functional deficit in diabetic cardiomyopathy. *Mol Cell Biochem* 1996; **160/161**: 283–288.
8. Koçak G, Aktan F, Canbolat O, *et al.* Alpha-lipoic acid treatment ameliorates metabolic parameters, blood pressure, vascular reactivity and morphology of vessels already damaged by streptozotocin-diabetes (The ADIC Study Group), *Diab Nutr Metab* 2000; **13**: 308–319.
9. Helen A, Vijayamal PL. Effect of vitamin A supplementation on cigarette smoke-induced lipid peroxidation. *Vet Hum Toxicol* 1997; **39**: 18–21.
10. Stohs SJ, Hassan MQ, Murray WJ. Effects of BHA, d-alpha-tocopherol and retinol acetate on TCDD-mediated changes in lipid peroxidation, glutathione peroxidase activity and survival. *Xenobiotica* 1984; **14**: 533–537.
11. Tuitoek PJ, Ziari S, Tsin AT, Rajotte RV, Suh M, Basu TK. Streptozotocin-induced diabetes in rats is associated with impaired metabolic availability of vitamin A (retinol). *Br J Nutr* 1996; **75**: 615–622.
12. Lu J, Dixon WT, Tsin AT, Basu TK. The metabolic availability of vitamin A is decreased at the onset of diabetes in BB rats. *J Nutr* 2000; **130**: 1958–1962.
13. Sakurai T, Tsuchiya S. Superoxide production from non-enzymatically glycated proteins. *FEBS Lett* 1988; **236**: 406–410.
14. Mak DHF, Ip SP, Li PC, Poon MKT, Ko KM. Alterations in tissue glutathione antioxidant system in streptozotocin-induced diabetic rats. *Mol Cell Biochem* 1996; **162**: 153–158.
15. Beatriz T, Bhagwan DG. Microsomal enzyme inducers and hypervitaminosis A in rats. *Arch Pathol Lab Med* 1976; **100**: 100–105.
16. Jain SK, Levine SN. Elevated lipid peroxidation and vitamin E-quinone levels in heart ventricles of streptozotocin treated diabetic rats. *Free Radical Biol Med* 1995; **18**: 337–341.
17. Spitz DR, Oberley LW. An assay for superoxide dismutase activity in mammalian tissue homogenates. *Anal Biochem* 1989; **179**: 8–18.
18. Aebi H. Catalase *in vitro*. *Methods Enzymol*. 1983; **105**: 121–126.
19. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun* 1976; **71**: 952–958.
20. Nierenberg DW, Lester DC. Determination of vitamins A and E in serum and plasma using a simplified clarification method and high-performance liquid chromatography. *J Chromatogr* 1985; **345**: 275–284.
21. Malvy DJ, Poveda JD, Debruyne M, Burtschy B, Dostalova L, Amedee-Manesme O. Immunonephelometry and radial immunodiffusion compared for measuring serum retinol-binding protein. *Eur J Clin Chem Clin Biochem* 1993; **31**: 47–48.

22. Giugliano D, Ceriello A, Paolisso G. Diabetes mellitus, hypertension, and cardiovascular disease: which role for oxidative stress? *Metabolism* 1995; **44**: 363–368.
23. Rauscher FM, Sanders RA, Watkins JB. Effects of new antioxidant compounds PNU-104067F and PNU-74389G on antioxidant defense in normal and diabetic rats. *J Biochem Mol Toxicol* 2000; **14**: 189–194.
24. Pieper GM, Jordan M, Dondlinger LA, Adams MB, Rosa AM. Peroxidative stress in diabetic blood vessels. Reversal by pancreatic islet transplantation. *Diabetes* 1995; **44**: 884–889.
25. Karasu Ç. Increased activity H_2O_2 in isolated aorta obtained from chronically streptozotocin-diabetic rats: effects of antioxidant enzymes and enzyme inhibitors. *Free Radic Biol Med* 1999; **27**: 16–27.
26. Karasu Ç. Time course of changes in endothelium-dependent and -independent relaxation of chronically diabetic aorta: role of reactive oxygen species. *Eur J Pharmacol* 2000; **392**: 163–173.
27. Ramanathan M, Jaiswal AK, Bhattacharya SK. Superoxide dismutase, catalase and glutathione peroxidase activities in the brain of streptozotocin induced diabetic rats. *Indian J Exp Biol* 1999; **37**: 182–183.
28. Van Dam PS, Bravenboer B, van Asbeck BS, van Oirschot JF, Marx JJ, Gispen WH. Effects of insulin treatment on endoneurial and systemic oxidative stress in relation to nerve conduction in streptozotocin-diabetic rats. *Eur J Clin Invest* 1996; **26**: 1143–1149.
29. Pereira B, Rosa LF, Safi DA, Bechara EJ, Curi R. Superoxide dismutase, catalase and glutathione peroxidase activities in the lymphoid organs of diabetic rats. *J Endocrinol* 1994; **142**: 161–165.
30. Sechi LA, Ceriello A, Griffin CA, *et al.* Renal antioxidant enzyme mRNA levels are increased in rats with experimental diabetes mellitus. *Diabetologia* 1997; **40**: 23–29.
31. Gey KF, Moser UK, Jordan P, Stahelin HB, Eichholzer M, Ludin, E. Increased risk of cardiovascular disease at suboptimal plasma concentration of essential antioxidants: an epidemiological update with special attention to carotene and vitamin C. *Am J Clin Nutr* 1993; **57**(Suppl.): 787S–797S.
32. Livrea MA, Tesoriere L, Bongiorno A, Pintaudi AM, Ciaccio M, Riccio A. Contribution of vitamin A to the oxidation resistance of human low density lipoproteins. *Free Radic Biol Med* 1995; **18**: 401–409.
33. Basu TK, Basualdo C. Vitamin A homeostasis and diabetes mellitus. *Nutrition* 1997; **13**: 804–806.
34. Ondreicka R, Beno I, Cerna O, *et al*. Relation between levels of vitamins C, E, A and beta-carotene and activity of antioxidant enzymes in the blood. *Bratisl Lek Listy* 1998; **99**: 250–254.

