

**Dehydroepiandrosterone administration counteracts oxidative imbalance and AGE formation in type 2 diabetic patients**

Enrico Brignardello<sup>1</sup>, MD, Cristina Runzo<sup>2</sup>, MD, Manuela Aragno<sup>3</sup>, PhD, Maria Graziella Catalano<sup>4</sup>, MD, Maurizio Cassader<sup>2</sup>, PhD, Paolo Cavallo Perin<sup>2</sup>, MD, and Giuseppe Boccuzzi<sup>1,4</sup>, MD.

<sup>1</sup>Oncological Endocrinology Unit, San Giovanni Battista Hospital, Turin, Italy

<sup>2</sup>Department of Internal Medicine, University of Turin, Italy

<sup>3</sup>Department of Experimental Medicine and Oncology, University of Turin, Italy

<sup>4</sup>Department of Clinical Pathophysiology, University of Turin, Italy

**Running title: DHEA and oxidative stress in type 2 diabetes**

**Corresponding author:**

Prof. Giuseppe Boccuzzi

Dipartimento di Fisiopatologia Clinica

Via Genova,3-10126 Torino (Italy)

E-mail: giuseppe.boccuzzi@unito.it

Received for publication 22 June 2007 and accepted in revised form 9 August 2007.

**Abstract**

**Objective:** Dehydroepiandrosterone (DHEA) has been shown to prevent oxidative stress in several “in vivo” and “in vitro” models. The study aimed to evaluate the effects of DHEA administration on oxidative stress, pentosidine concentration and TNF $\alpha$  / TNF $\alpha$  receptor system activity in patients with type 2 diabetes.

**Research Design and Methods:** Twenty patients were enrolled in the study and randomly assigned to the DHEA (n = 10) or placebo (n = 10) group. Twenty healthy sex- and age-matched subjects with normal glucose levels served as controls. DHEA was given as a single daily dose of 50 mg, for 12 weeks.

**Results:** Oxidative stress parameters were significantly higher in diabetic patients versus controls. Pentosidine levels, as well as sTNF-RI and sTNF-RII, were also higher in diabetic patients. After DHEA, plasma levels of ROS and HNE dropped by 53% and 47% respectively, whereas the non-enzymatic antioxidants GSH and Vitamin E increased (+38 and +76%, respectively). The same changes in oxidative parameters were detected in peripheral blood mononuclear cells (PBMC). DHEA treatment also induced a marked decrease of pentosidine plasma concentration in diabetic patients (-50%). Moreover, the TNF $\alpha$  / TNF $\alpha$  receptor system was shown to be less activated after DHEA treatment, both in plasma and in PBMC.

**Conclusions:** Data indicate that DHEA treatment ameliorates the oxidative imbalance induced by hyperglycaemia, down-regulates the TNF $\alpha$  / TNF $\alpha$  receptors system and prevents AGE formation, suggesting a beneficial effect on the onset and/or progression of chronic complications in type 2 diabetic patients.

The onset and progression of diabetes complications involves a complex interplay between ranges of pathogenic mechanisms. However, emerging evidence suggests that a single early phenomenon, i.e. the overproduction of superoxide by the respiratory chain, plays a key role in the pathogenesis of both microvascular and macrovascular chronic complications (1-3).

The production of advanced glycated end-products (AGE) is among the main mechanisms recruited by oxidative stress and involved in the pathogenesis of tissue injury (3,4). AGEs progressively accumulate with time at the sites of diabetic microvascular disease and mediate tissue damage by activation of specific receptors at distant sites, via the circulation (3,5). Moreover the AGE/AGE receptors interaction, along with hyperglycemia-induced oxidative stress, possibly serves as a key activator of upstream kinases, leading to increased production of inflammatory cytokines thought to be involved in the progression of diabetic chronic complications (6).

Interruption of free radical overproduction by antioxidants counteracts AGE formation (2). Nevertheless, despite convincing experimental results, clinical trials with traditional antioxidants have been disappointing (7): the activity of the antioxidants used in those trials is limited to scavenging already-formed oxidants and is stoichiometric. A compound of physiological origin that possesses multi-targeted antioxidant properties is dehydroepiandrosterone (DHEA), a multifunctional steroid that has been shown to prevent tissue damage induced by hyperglycaemia in several “in vivo” and “in vitro” models (8,9). It also prevents the up-regulation of AGE receptors observed in the hippocampus of streptozotocin-treated diabetic rats (10).

The study aimed to examine the effects of DHEA administration on oxidative stress, pentosidine (a marker of AGE-biogenesis) concentration and TNF- $\alpha$ /TNF- $\alpha$  receptors in patients with recently-diagnosed type 2 diabetes, controlled with diet alone and without any evidence of chronic complications, i.e. with the disease at a very early stage.

## **Research Design and Methods**

### Subjects

The study was approved by the Ethics Committee at our Institution and written informed consent was obtained from all recruited subjects. It was a randomized, double-blind, placebo-controlled small-scale study of 12-wk duration.

The study group comprises 20 patients with recently-diagnosed type 2 diabetes treated with diet alone, having good glycemic control and taking no drugs potentially interfering with redox status. All patients were non-smokers and showed no evidence of chronic diabetic complications.

The control group comprised 20 healthy subjects, matched by sex and age, with normal glucose levels.

### Study design

After consent, patients were randomly assigned to the DHEA (n = 10) or the placebo (n = 10) group. DHEA was purchased from DHEA Pharma® (Miami, FL, USA) and given as a single daily oral dose of 50 mg at 8:00 h for 12 weeks. The 10 patients enrolled in the placebo group received pills that were identical in appearance to the DHEA formulation. Compliance was checked by pill counts. At baseline and at the end of the treatment all patients were subjected to complete physical examination, and fasting blood samples were collected to evaluate

oxidative stress parameters, pentosidine levels and TNF $\alpha$ /TNF receptors.

#### Serum DHEA, DHEAS, glucose, serum insulin, HOMA, HbA<sub>1c</sub>

Serum DHEA and DHEA sulphate (DHEAS) were determined by specific RIAs (Diagnostic System Laboratories, Oxford, UK). Fasting serum glucose was measured by the glucose oxidase method (HITACHI 911 Analyser, Sentinel Ch., Milan, Italy), and fasting serum insulin by immunoradiometric assay (Radim S.p.A., Pomezia, Italy). HbA<sub>1c</sub> was determined by standardized affinity high performance liquid chromatography (Bio Rad, Italy). The HOMA insulin resistance index was calculated as the product of basal glucose and insulin levels divided by 22.5 (11).

#### Cytosol extracts from PBMCs

To isolate Peripheral Blood Mononuclear Cells (PBMC), heparin-anticoagulated blood was subjected to density-gradient centrifugation on Lymphoprep<sup>TM</sup> (Fresenius Kabi Norge AS, Oslo, Norway). The cytosolic extracts were prepared as reported elsewhere (12). PBMCs were resuspended in buffer containing 20 mM HEPES, pH 7.9, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1% NP-40, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 5  $\mu$ g/ml aprotinin, 2.5  $\mu$ g/ml leupeptin, then centrifuged at 1,000 g for 5 min at 4°C. Supernatants were centrifuged at 105,000 g at 4°C for 40 min to obtain cytosolic fraction and protein content was determined.

#### Pentosidine

Plasma samples (200  $\mu$ l) were treated with 6 M hydrochloric acid for 2 hours at 40°C and centrifuged (4000 rpm); 20  $\mu$ l of supernatant were injected.

A Thermo-Finnigan Surveyor instrument (Thermo Electron, Rodano, Italy),

equipped with autosampler and PDA-UV 6000 LP detector, was used. Mass spectrometry analyses were performed using a LCQ Deca XP plus spectrometer, with electrospray interface and ion trap as mass analyzer.

The chromatographic separations were run on a Varian Polaris C18-A column (150  $\times$  2 mm, 3  $\mu$ m particle size) (Varian, Leini, Italy). Flow rate 200  $\mu$ L min<sup>-1</sup>. Gradient mobile phase composition was adopted: 95/5 to 0/100 v/v 5 mM heptafluorobutanoic acid in water/methanol in 13 min.

The LC column effluent was delivered to UV detector (200-400 nm) and then to the ion source using nitrogen as sheath and auxiliary gas (Claind Nitrogen Generator apparatus, Lenno, Italy). The tuning parameters adopted for the ESI source were as follows: source current 80.00  $\mu$ A, capillary voltage 3.00 V, tube lens offset 15 V; for ions optics, multipole 1 offset – 5.25 V, inter multipole lens voltage -16.00 V, multipole 2 offset -9.00 V. Mass spectra were collected in tandem MS mode: MS<sup>2</sup> of (+) 379 *m/z* with 33 % CE in the range 100-400 *m/z*.

#### Oxidative biochemical parameters

Reduced glutathione (GSH), Hydroxynonenal (HNE) and Reactive Oxygen Species (ROS) levels were evaluated as reported elsewhere (12). For GSH levels, a mixture was directly prepared in cuvette: 2.25 ml of 0.1 M K-phosphate buffer, pH 8.0; 0.2 ml of the sample (plasma or PBMCs cytosol fractions); 25  $\mu$ l of Ellman's reagent (10 mM DTNB in methanol). After 1 minute the assay absorbance was measured at 412 nm and the GSH concentration was calculated by comparison with a standard curve.

HNE was determined on plasma or PBMCs cytosol fractions. An aliquot of cytosol (0.5 ml) was added to an equal

volume of acetonitrile:acetic acid (96:4, v/v). After centrifugation, the supernatant was injected into an HPLC (Waters Associated, Milford, MA, USA) Symmetry C<sub>18</sub> column (5 mm, 3.9x150 mm). The mobile phase was acetonitrile:bidistilled water (42%,v/v). The HNE concentration was calculated by comparison with a standard solution of HNE of known concentration.

ROS were measured in plasma or PBMCs cytosol fractions using probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is a stable, non-fluorescent molecule that readily crosses the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent 2',7'-dichlorofluorescein (DCFH), which is rapidly oxidized, in the presence of peroxides, to highly fluorescent 2',7'-dichlorofluorescein (DCF); DCF is measured fluorimetrically. Alpha-tocopherol (vitamin E) was assayed using the method described by Burton (13): after extraction of 0.5 ml of plasma with 1ml n-heptane and centrifugation, the heptane phase was collected for HPLC analysis. A Supercosil-LC-Si column (25 cm x 4.6mm, Supelco Inc., PA, USA) was used, the mobile phase being n-hexane-isopropanol (99:1, v:v) and the flow rate 2.0 ml /min; the fluorescence detector was set to 298 nm excitation and 325 nm emission.

#### TNF $\alpha$ , TNF-RI and TNF-RII

##### *Serum levels*

Serum levels of Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ), its soluble receptors TNF-RI (sTNF-RI) and TNF-RII (sTNF-RII) were measured using ELISA tests (for TNF $\alpha$ , Immunotech, Marseille, France; for sTNF-RI and sTNF-RII, Quantikine HS, R&D System Inc, Minneapolis MN, USA), following manufacturer's instructions.

#### *Real-time RT-PCR*

Total RNA was extracted from PBMC using TRIzol Reagent (Invitrogen, Groningen, The Netherlands), as reported elsewhere (12). DNase was added to remove remaining genomic DNA. RNA was reverse-transcribed using ISCRIP T cDNA SYNTHESIS KIT (BioRad Laboratories, Inc.).

Primers were designed using the Beacon 5 program. Primers: for TNF $\alpha$ , forward 5'-CGC CAC CAC GCT CTT CTG C, reverse 5'-GGG CTA CAG GCT TGT CAC TCG; for TNF-RI, forward 5'-CTG CCA GGA GAA ACA GAA CAC C, reverse 5'-GCG TCC TCA GTG CCC TTA ACA TTC; for TNF-RII: forward 5'-CGG TGT GGG CTG TGT CGT AG, reverse 5'- GAG GCT GCG GCT GTG GAG; for  $\beta$ -actin, forward 5'-GCG AGA AGA TGA CCC AGA TC, reverse 5'-GGA TAG CAC AGC CTG GAT AG.

Real-time PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories, Inc.) with SYBR green fluorophore. Reactions were run in a total volume of 25  $\mu$ l-included 12.5  $\mu$ l IQ SYBR Green Supermix (BioRad Laboratories, Inc.), 1  $\mu$ l of each primer at 10  $\mu$ M concentration, and 5  $\mu$ l of the reverse-transcribed cDNA template. The protocol is as follows: denaturation (95°C for 5 min), amplification repeated 40 times (95°C for 15 sec, 60°C for 1 min). A melt curve analysis was performed to ensure a single amplified product for every reaction. All reactions were carried out in at least triplicate. Analysis of relative gene expression was performed using Gene Expression Macro<sup>TM</sup> software (Biorad Laboratories, Inc.).

#### Statistical analysis

Results are presented as means  $\pm$  SE. Differences between means were analyzed for significance using the Student's *t* test.

A  $p$  value  $< 0.05$  was considered significant.

## **Results**

Oxidative stress was significantly higher in diabetic patients than controls, as demonstrated by higher levels of ROS and HNE and reduced levels of GSH and Vitamin E (Table 1). Pentosidine levels, as well as sTNF-RI and sTNF-RII, were also higher in diabetic patients than in controls, whereas serum TNF- $\alpha$  levels were within the normal range (Table 1).

### Effects of DHEA on BMI and glycemic control

DHEA treatment had no effect on patient's body mass index (BMI) of the patients. Basal glucose concentration, HbA<sub>1c</sub> levels and HOMA index were also unaffected (Table 2).

### Effects of DHEA on oxidative state

After DHEA treatment, DHEA and DHEAS levels were significantly increased ( $P < 0.05$ , Table 3). Oxidative stress parameters were significantly modified by DHEA treatment, both in plasma and in PBMC. Plasma levels of ROS dropped by 53% (Table 3 and Figure 1, panel A). Likewise, a 47% reduction of plasma HNE was observed after DHEA treatment (Table 3 and Figure 1, panel A), whereas plasma levels of the non-enzymatic antioxidants GSH and Vitamin E increased by 38 and 76%, respectively (Table 3 and Figure 1, panel A). The same trends were found in PBMC (Table 3 and Figure 1, panel B). No correlation was found between DHEA or DHEAS levels and both the oxidative and the anti-oxidant parameters either before or after DHEA treatment.

### Effects of DHEA on pentosidine levels

DHEA treatment markedly decreased pentosidine plasma concentration in

diabetic patients (- 50%; Table 3 and Figure 1, panel A).

### Effects of DHEA on TNF $\alpha$ and TNF $\alpha$ receptors

After DHEA treatment, no modification of serum TNF $\alpha$  and sTNF-RI was observed (Table 3), whereas in PBMC their mRNA expression was reduced by 33 and 29%, respectively (Figure 1, panel C). On the contrary, serum sTNF-RII levels were reduced after DHEA treatment (Table 3), without any modification in mRNA expression (Figure 1, panel C).

All the above parameters were unchanged in diabetic patients treated with placebo (data not shown).

## **Discussion**

Type 2 diabetic patients with good glycemic control and with no evidence of chronic diabetic complications - like those enrolled in this study - show a redox imbalance characterized by increased production of highly reactive oxygen species and lower than normal antioxidant potential. We show here that DHEA treatment counteracts this oxidative imbalance: after 12 weeks, the concentrations of ROS and HNE were greatly reduced both in plasma and in the cytosol of PBMC, whereas levels of the non-enzymatic antioxidants GSH and Vitamin E were increased. These results are in agreement with the multi-targeted antioxidant effect of DHEA previously reported by our group (9,10,12). We couldn't find any correlation between serum DHEA or DHEAS levels and both the oxidative and the anti-oxidant parameters either before or after DHEA treatment. This result is not surprising, since DHEA is not a scavenger compound acting in a stoichiometric manner and exerts its anti-oxidants effects in a complex and non completely defined way (10,12).

Moreover, DHEA treatment significantly reduced the plasma concentration of pentosidine in these patients, in line with its effects on AGE and AGE receptors, which have been reported in experimental diabetes (12). AGEs, whose production is triggered by oxidative stress, are clearly implicated in the development and progression of chronic diabetic complications (3,4). Among AGEs, pentosidine is a well-characterized compound and is used as a marker of AGE- biogenesis (14); it is considered a good predictor for the development of microvascular complications in diabetic patients (15). Interestingly, DHEA reduces pentosidine concentration in type 2 diabetic patients without any influence on glycemic control, strongly suggesting that this effect can involve its ability to improve redox balance.

Hyperglycemia-induced oxidative stress, may, either directly or through the AGE/AGE receptor interaction, serve as a key activator of upstream kinases, leading to an increase in the plasma inflammatory cytokine concentrations that is thought to be involved in the progression of chronic diabetic complications (6) as well as in the development of insulin resistance (16).

Compared to controls, diabetic patients showed higher levels of sTNF-RI and sTNF-RII, whereas TNF $\alpha$  plasma concentrations were similar. After binding of TNF $\alpha$  to its receptors, cleavage of the extracellular parts elicits the soluble forms, known as sTNF-RI and sTNF-RII, which are thought to reflect the degree of TNF system activation (17). TNF $\alpha$  is not a very stable protein, and its serum level may not adequately reflect its activity. On the contrary, serum concentration of soluble TNF $\alpha$  receptors have been shown to be increased in diabetic patients (18) and the level of sTNF-RII has been considered the best predictor of TNF $\alpha$  system activation as well as being a

marker of insulin resistance (19). DHEA treatment reduced the serum concentration of sTNF-RII and mRNA expression of TNF $\alpha$  and TNF-RI in PBMC. A reduction in mRNA expression of TNF $\alpha$  indicates a down-regulation of this system, which is reflected by a concomitant reduction in TNF-RI expression. As far as TNF-RII is concerned, our observation of a reduction in its serum levels without any change in its mRNA expression implies reduced shedding of the protein, suggesting improved insulin sensitivity after DHEA treatment. A relationship between TNF $\alpha$  system and insulin resistance has indeed been reported (20), as has a negative correlation between TNF-RII shedding and insulin sensitivity (21). Moreover reactive oxygen species, which are markedly reduced by DHEA treatment, have recently been reported to have a causal role in insulin resistance (16). In accordance with previous observations (22), the present data suggest that DHEA treatment might influence insulin sensitivity in type 2 diabetic patients, through its effects on the TNF $\alpha$  system, despite the absence of HOMA index modifications that we observed.

In conclusion these data, together with the experimental data from rodents, suggest that DHEA treatment might prevent many of the events that lead to cellular damage induced by hyperglycemia, thus counteracting the onset and/or the progression of chronic complications in type 2 diabetic patients. Of interest, this result was obtained in the absence of any improvement of glycemic control. Present management of hyperglycemia is based upon the assumption that the best way to reduce the risk of diabetic complications is to achieve optimal glycemic control. However, it should be pointed out that patients receiving intensive therapy designed to achieve glycemic control still

develop diabetic complications, even though their prevalence is reduced (23). If hyperglycemia cannot be effectively prevented, the only way to impede diabetic complications will be to interrupt the pathways that lead from hyperglycemia to target organ damage. AGEs clearly represent one such pathway. A similar preventive activity against hyperglycaemia-induced oxidative stress has been postulated for drugs such as statins, angiotensin-converting enzyme inhibitors, angiotensin II type 1 receptor blockers, calcium channel blockers and thiazolidinediones, and their clinical use to prevent chronic complications in

diabetic patients has been suggested (24). Compared to these drugs DHEA has the advantage to be a physiologic steroid without side effects at the dosage used in this study. The usefulness of this novel approach to protect diabetic patients against tissue damage appears to be worth further exploration through multicenter clinical trials.

#### **Acknowledgements**

This study was supported by the Special Project 'Oncology', Compagnia San Paolo, Turin, by MIUR and by Regione Piemonte.

## **References**

1. Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40:405-412, 1991
2. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardini I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787-790, 2000
3. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001
4. Vlassara H: The AGE-receptor in the pathogenesis of diabetic complications. *Diabetes Metab Res Rev* 17:436-443, 2001
5. Forbes JM, Yee LT, Thallas V, Lassila M, Candido R, Jandeleit-Dahm KA, Thomas MC, Burns, WC, Deemer EK, Thorpe SM, Cooper ME, Allen TJ: Advanced glycation end product interventions reduce diabetes-accelerated atherosclerosis. *Diabetes* 53:1813-1823, 2004
6. Esposito K, Nappo F, Marfella R, Giugliano G, Giugliano F, Ciotola M, Quagliaro L, Ceriello A, Giugliano D: Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans. Role of oxidative stress. *Circulation* 106:2067-2072, 2002
7. Lonn E, Yusuf S, Hoogwerf B, Pogue J, Yi Q, Zinman B, Bosch J, Gagenais G, Mann JFE, Gerstein HC: Effects of vitamin E on cardiovascular and microvascular outcomes in high-risk patients with diabetes. *Diabetes Care* 25:1919- 1927, 2002
8. Brignardello E., Beltramo E, Molinatti PA, Aragno M, Gatto V, Tamagno E, Danni O, Porta M, Boccuzzi G: Dehydroepiandrosterone protects bovine retinal capillary pericytes against glucose toxicity. *J Endocrinol* 158:21-26, 1998
9. Aragno M, Parola S, Brignardello E, Mauro A, Tamagno E, Manti R, Danni O, Boccuzzi G: Dehydroepiandrosterone prevents oxidative injury induced by transient ischemia/reperfusion in the brain of diabetic rats. *Diabetes* 49:1924-1931, 2000
10. Aragno M, Mastrocola R, Medana C, Restivo F, Catalano MG, Pons N, Danni O, Boccuzzi G: Up-regulation of advanced glycated products receptors in the brain of diabetic rats is prevented by antioxidant treatment. *Endocrinology* 146:561-5567, 2005
11. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28:412-419, 1985
12. Aragno M, Mastrocola R, Medana C, Catalano MG, Vercellinatto I, Danni O, Boccuzzi G: Oxidative stress-dependent impairment of cardiac-specific transcription factors in experimental diabetes. *Endocrinology* 147:5967-5974, 2006
13. Burton GW, Traber MG, Acuff RV, Walters DN, Kayden H, Hughes L, Ingold KU. Human plasma and tissue alpha-tocopherol concentrations in response to supplementation with deuterated natural and synthetic vitamin E. *Am J Clin Nutr* 67:669-684, 1998
14. Raj DSC, Choudhury D, Welbourne TC, Levi M: Advanced glycation end products: a nephrologist's perspective. *Am J Kidney Disease* 35:365-380, 2000
15. Monnier VM, Bautista O, Kenny D, Sell DR, Fogarty J, Dahms W, Cleary PA, Lachin J, Genuth S: Skin collagen glycation, glycooxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes: relevance of glycated collagen products versus HbA1c as markers of diabetic complications. DCCT Skin Collagen Ancillary Study Group. *Diabetes Control and Complications Trial. Diabetes* 48:870-880, 1999

16. Houstis N, Rosen ED, Lander ES: Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 440:944-948, 2006
17. Nophar Y, Kemper O, Brakebusch C, Englemann H, Zwang R, Aderka D, Holtmann H, Wallach D: Soluble forms of tumor necrosis factor receptors (TNF-Rs). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. *EMBO J* 9:3269-3278, 1990
18. Fernandez-Real JM, Brich M, Ricart W, Casamitjana R, Gutierrez C, Vendrell J, Richart C: Plasma levels of the soluble fraction of tumor necrosis factor receptor 2 and insulin resistance. *Diabetes* 47:1757-1762, 1998
19. Hotamisligil GS, Arner P, Atkinson RL, Spiegelman BM: Differential regulation of the p80 tumor necrosis factor receptor in human obesity and insulin resistance. *Diabetes* 46:451-455, 1997
20. Winkler G, Lakatos P, Salamon F, Nagy Z, Speer G, Kovacs M, Harnos G, Dworak O, Cseh K: Elevated serum TNF-alpha level as a link between endothelial dysfunction and insulin resistance in normotensive obese patients. *Diabet Med* 16:207-211, 1999
21. Fernandez-Real JM, Lainez B, Vendrell J, Rigla M, Castro A, Penarroja G, Broch M, Perez A, Richart C, Engel P, Ricart W: Shedding of TNF-alpha receptors, blood pressure, and insulin sensitivity in type 2 diabetes mellitus. *Am J Physiol Endocrinol Metab* 282:E952-959, 2002
22. Kawano H, Yasue H, Kitagawa A, Hirai N, Yoshida T, Soejima H, Miyamoto S, Nakano M, Ogawa H: Dehydroepiandrosterone supplementation improves endothelial function and insulin sensitivity in men. *J Clin Endocrinol Metab* 88:3190-3195, 2003
23. Soro-Paavonen A, Forbes JM: Novel therapeutics for diabetic micro- and macrovascular complications. *Curr Med Chem* 13:1777-1788, 2006
24. Ceriello A. Controlling oxidative stress as a novel molecular approach to protecting the vascular wall in diabetes. *Curr Opin Lipidol* 17:510-518, 2006

**Table 1:** BMI, serum concentration of glucose, ROS, GSH, vitamin E, HNE, pentosidine and cytokines in the study group at baseline. Means  $\pm$  SE.

Statistical analysis was performed using the unpaired data Student's "t" test.

	<b>Controls (n=20)</b>	<b>Diabetic patients (n=20)</b>	<b>P value</b>
<b>BMI</b>	23.50 $\pm$ 1.12	25.50 $\pm$ 1.14	n.s.
<b>Glucose (mg/ml)</b>	78.00 $\pm$ 4.20	131.00 $\pm$ 7.70	< 0.001
<b>ROS (UF/ml)</b>	111.9 $\pm$ 5.2	353.3 $\pm$ 42.3	< 0.001
<b>GSH (<math>\mu</math>g/ml)</b>	91.6 $\pm$ 7.4	56.9 $\pm$ 9.0	< 0.001
<b>Vitamin E (<math>\mu</math>M)</b>	126.2 $\pm$ 8.5	65.8 $\pm$ 5.3	< 0.001
<b>HNE (<math>\mu</math>M)</b>	1.65 $\pm$ 0.21	5.15 $\pm$ 0.81	< 0.05
<b>Pentosidine (<math>\mu</math>M)</b>	0.75 $\pm$ 0.06	2.44 $\pm$ 0.18	< 0.001
<b>TNF<math>\alpha</math> (pg/ml)</b>	2.34 $\pm$ 0.50	3.91 $\pm$ 1.00	ns
<b>sTNF-RI (pg/ml)</b>	1039 $\pm$ 94	1584 $\pm$ 100	< 0.001
<b>sTNF-RII (pg/ml)</b>	1792 $\pm$ 147	3136 $\pm$ 291	< 0.001

**Table 2:** Characteristics of patients at baseline and after DHEA (n = 10) or placebo (n = 10) treatment. Means  $\pm$  SE.

	<b>DHEA (50 mg/die)</b>		<b>Placebo</b>	
	<b>Baseline</b>	<b>12 weeks</b>	<b>Baseline</b>	<b>12 weeks</b>
<b>Age</b>	63.9 $\pm$ 11.1		58.0 $\pm$ 16.77	
<b>BMI</b>	25.7 $\pm$ 1.1	25.4 $\pm$ 1.4	25.7 $\pm$ 1.5	25.1 $\pm$ 1.4
<b>PAOS (mm Hg)</b>	127.7 $\pm$ 4.6	127.7 $\pm$ 3.0	137.0 $\pm$ 2.8	132.0 $\pm$ 2.7
<b>Plasma glucose (mmol/l)</b>	7.32 $\pm$ 0.37	7.40 $\pm$ 0.52	6.63 $\pm$ 0.81	6.36 $\pm$ 0.71
<b>Creatinine (<math>\mu</math>mol/l)</b>	78.7 $\pm$ 3.8	84.9 $\pm$ 5.9	81.3 $\pm$ 3.5	82.2 $\pm$ 2.9
<b>LDL cholesterol (mmol/l)</b>	3.00 $\pm$ 0.22	3.17 $\pm$ 0.27	3.82 $\pm$ 0.14	3.53 $\pm$ 0.31
<b>HbA1C (%)</b>	6.5 $\pm$ 0.2	6.8 $\pm$ 0.3	6.4 $\pm$ 0.5	6.7 $\pm$ 0.6
<b>HOMA index</b>	11.8 $\pm$ 1.9	12.7 $\pm$ 2.2	12.4 $\pm$ 2.0	11.3 $\pm$ 2.8

**Table 3:** Effects of DHEA treatment on DHEA, DHEAS, oxidative and antioxidant parameters, and TNF $\alpha$  / TNF $\alpha$  receptor system in plasma and / or PBMC. Mean  $\pm$  SE, n = 10. Statistical analysis was performed using the paired data Student's "t" test.

	<b>DHEA (50 mg/die)</b>					
	<b>P L A S M A</b>			<b>P B M C</b>		
	<b>Baseline</b>	<b>12 weeks</b>	<b>P value</b>	<b>Baseline</b>	<b>12 weeks</b>	<b>P value</b>
<b>ROS (UF/ml)</b>	372.3 ± 66.6	183.0 ± 12.1	< 0.05	307.4 ± 40.7	186.7 ± 37.1	< 0.01
<b>HNE (µM)</b>	6.40 ± 0.81	3.17 ± 0.42	< 0.001	5.09 ± 0.27	2.60 ± 0.41	< 0.001
<b>GSH (µg/ml)</b>	60.7 ± 2.0	85.7 ± 2.4	< 0.01	314.4 ± 53.7	534.1 ± 73.8	< 0.01
<b>DHEA (nM)</b>	24.5 ± 2.7	32.8 ± 2.8	<0.05	n.d.	n.d.	
<b>DHEAS (µM)</b>	1.7 ± 0.2	2.2 ± 0.1	<0.05	n.d.	n.d.	
<b>Vitamin E (µM)</b>	68.3 ± 5.4	120.3 ± 10.9	< 0.01	n.d.	n.d.	
<b>Pentosidine (µM)</b>	2.31 ± 0.13	1.31 ± 0.06	< 0.001	n.d.	n.d.	
<b>TNFα (pg/ml)</b>	3.10 ± 1.40	6.88 ± 1.62	ns	n.d.	n.d.	
<b>TNF-RI (pg/ml)</b>	1602 ± 115	1591 ± 112	ns	n.d.	n.d.	
<b>TNF-RII (pg/ml)</b>	3440 ± 365	2829 ± 471	< 0.01	n.d.	n.d.	

**Figure Legend**

**Figure 1. Effects of DHEA on variation of oxidative stress parameters, TNF $\alpha$  and TNF $\alpha$  receptors.** Panel A: Changes in levels of ROS, GSH, vitamin E, HNE and pentosidine in plasma of diabetic patients treated with DHEA for 12 wks. Panel B: Changes in levels of ROS, GSH and HNE in PBMC of diabetic patients treated with DHEA for 12 wks. Panel C: Changes in mRNA expression of TNF $\alpha$ , TNF-RI and TNF-RII in PBMC of diabetic patients treated with DHEA for 12 wks. Variation is expressed as percentage with respect to levels at baseline. Significance vs baseline: \* P<0.05, \*\* P<0.01, \*\*\* P<0.001.

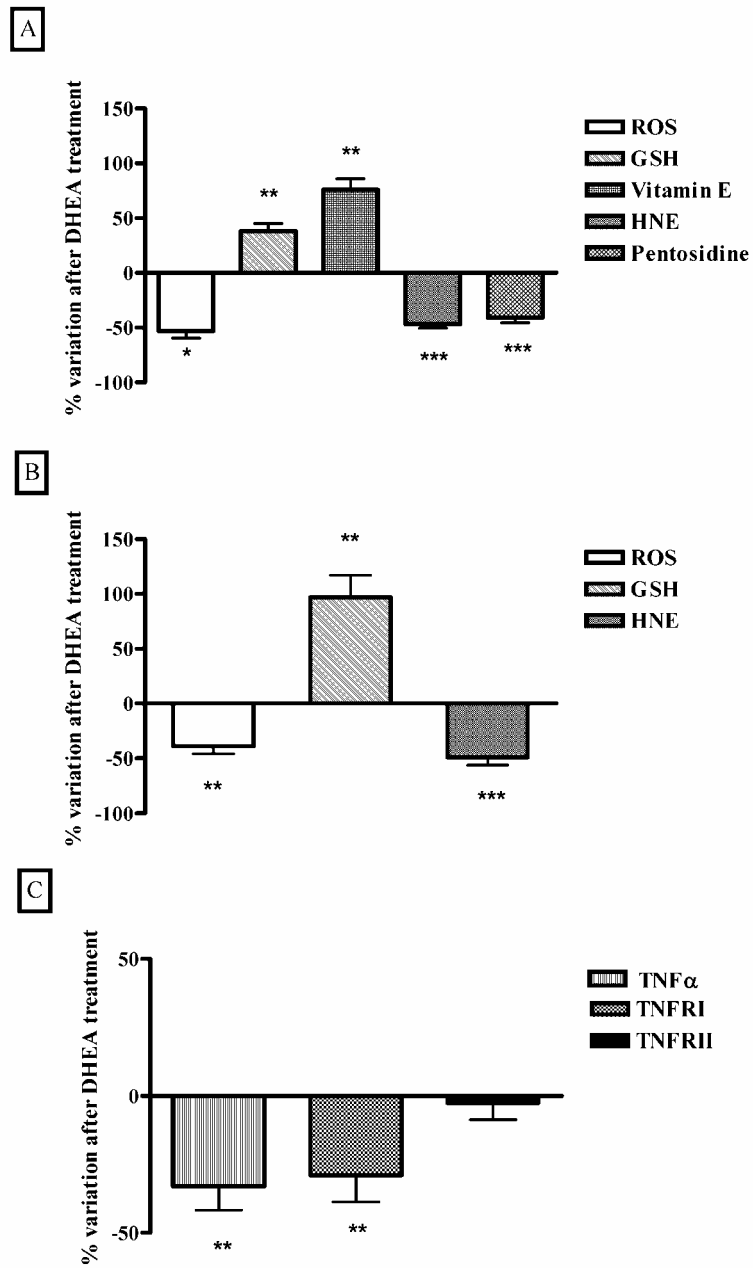


Figure 1