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Evaluation of Oxidative Stress and the Efficacy of Antioxidant Treatment in Diabetes Mellitus

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1. Introduction

Studies on the efficacy of antioxidant treatment in type 1 diabetes mellitus is an interesting, actual research subject. Reactive oxygen species (ROS) are continuously produced and eliminated by living organisms normally maintaining ROS at certain steady-state levels. Under some circumstances, the balance between ROS generation and elimination is disturbed leading to enhanced ROS level causing oxidative stress [Lushchak, 2011]. Oxidative stress is involved in the development of several important diseases (cancer, ulcer, atherosclerosis, autoimmune diseases, ischaemia-reperfusion injury, emphysema, inflammation, etc.).

Oxidative stress due to increased production of reactive oxygen species and/or impaired antioxidant capacity of the body plays a special, important role in the development of type 1 diabetes mellitus and its complications [Baynes, 1991], [Giugliano et al., 1995], [Krippeit-Drews et al., 1994], [Nakazaki et al., 1995], [Nomikos et al., 1989], [Raes et al, 1995], [Shinn, 1998].

Under normal conditions, oxidative tissue damage is prevented by enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are three enzymes involved in detoxification of reactive oxygen species (superoxide radical, hydroxyl radical, and hydrogen peroxide). Pancreatic cells have a poor antioxidant defence system, so they are very vulnerable to oxidative stress, especially to superoxide mediated radical damage [Dejica, 2000].

Evaluation of oxidative status can be made by various methods, most of them very sophisticated, requiring laboratories with modern equipment, and are used almost exclusively for research purposes.

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Exploring the body oxidative status can be made through the following ways:

1. Free radical measurement by absorption spectroscopy with electronic spin resonance (ESR) and electronic paramagnetic resonance (EPR) [Olinescu, 1994].
2. Measurement of chemical uptake (chemical trapping) by quantitative determination of the elimination of specific derivatives of salicylic acid, hydroxylated or nitrosylated compounds.
3. Measuring the antioxidant capacity of each antioxidant in part or total plasma antioxidant capacity.
4. Determination of antioxidant enzyme activities (SOD, CAT, GPX) and non-enzymatic antioxidants (tocopherols and tocotrienols, vitamin C, ubiquinone, glutathione, carotenoids and vitamin A, bilirubin, melatonin, uric acid, ceruloplasmin, vitamin K, lipoic acid) [Dejica, 2000, 2001].
5. The measurement of biological compounds resulting from oxidative processes:
 - lipid compounds - conjugated dienes, hydroperoxides, aldehydes (malondialdehyde, hydroxynonenal)
 - ethane and pentane measurements in expired gases
 - DNA beta-hydroxydeoxyguanosine
 - protein derivatives (carbonyl or thiol groups)
 - amino acids - methionine sulfoxide, ortho-tyrosine, dityrosine, nitrotyrosine, chlorotyrosine.
6. Measurement of the antioxidants / oxidizing substances ratio (eg. ascorbic acid / dehydroascorbic acid and reduced glutathione / oxidized glutathione) [Prior & Cao, 1999].

2. Antioxidants

Determination of trace elements is also of interest because some are in the active center of antioxidant enzymes: for example Cu, Zn and Mn are found in the structure of SOD, and Se is present in the active center of GPX, but also possesses antioxidant effect independent of this enzyme [Olinescu, 1994].

The variety of antioxidant substances in the body, the difficulty of measuring their individual level and the interactions between them require methods for measuring total antioxidant capacity (TAOC) in different biological samples [Cadenas & Packer, 2002].

By measuring TAOC one can assess not only the interaction effects of antioxidants known, but also the antioxidant action of unidentified components present in human plasma. In most methods uric acid is the major contributor to the TAOC of plasma, so increasing plasma levels of uric acid can mask the depletion of ascorbate or other antioxidants in some pathological conditions if only TAOC measurement is carried out.

TAOC are methods used to measure indirect inhibition involving a prooxidant (typically a free radical) and an oxidizable substrate. The prooxidant induces oxidative deterioration of the substrate, which is inhibited in the presence of the antioxidant.

Antioxidant capacity can be defined as the ability of a compound to reduce prooxidants. In biological systems, prooxidants are usually defined as toxic substances causing oxidative damage to lipids, proteins, nucleic acids, leading to a variety of pathological events, but not

every prooxidant is necessary a toxic compound. Antioxidants are substances that, in low concentrations compared to an oxidizable substrate, prevent or delay oxidation initiated by a prooxidant [Dejica, 2001].

Several vitamins are well known for their antioxidant properties: vitamin C exhibits its effect in hydrophilic environment, while vitamin A and E protect especially the cell membranes, and act in hydrophobic phase. Many other, more complex antioxidant substances are found in plants.

3. Implication of oxidative stress and glycation process in diabetes mellitus

In type 2 diabetic patients, besides the relative insulin deficiency, there is a certain grade of insulin resistance. The relationship between reactive oxygen species and the effect of insulin has been studied, and the results showed that in elderly people, presenting intense exposure to oxidative stress, the ratio between GSH/GSSG is reduced, leading to intensified lipoperoxidation. This phenomenon might exhibit a negative influence on the integrity of plasma membranes, leading to their disfunction, regarding for instance the transmembrane glucose transport. There is a high probability that the peripheral action of insulin is disturbed by the negative effect of reactive oxygen species on membrane ATP-ase activity [Dejica, 2000].

Peroxidation of lipids (especially LDL) plays an important role in inducing macrovascular lesions found in both diabetes and in atherosclerotic disease. Susceptibility to oxidation of lipoproteins seems to be a key element in the initiation and propagation of the atherogenic process [Dobreanu M., Módy, 1998]. In diabetic patients, lipid oxidation affects circulating lipids, and also those present in cell membranes or myelin layers. Hyperglycemia, excessive autooxidation and decreased antioxidant capacity registered in diabetic patients are responsible for intensified oxidative processes.

The speed of non-enzymatic glycation is proportional to the blood sugar level. Glucose (and fructose and galactose) are attached to the extreme N-terminal peptide chain, initially forming a Schiff base (aldimine), which is unstable, then by Amadori rearrangement a stable cetoimine is formed, and advanced glycation end products, leading to alterations in configuration, of the electronegative charge and molecular recognition of proteins.

Amadori-products can also be oxidized, by reactions catalyzed by transition metals, releasing eritronic acid and forming carboxymethylated lysine. Its level is double in the collagen of the skin of diabetic patients compared to non-diabetic subjects, and it is positively correlated with the presence of retino- and nephropathy in diabetic patients [Wolff, 1993].

All structural proteins and those circulating in the body can suffer non-enzymatic glycation processes. They can alter protein structure and function of vessels, nerves, liver, skin and other organs. Glycosylation of LDL lipoprotein particles decreases their catabolism and accelerates HDL catabolism, disorders that may explain in part the modifications present in macroangiopathy. Glycosylated proteins are more susceptible to attack by reactive oxygen species.

Enzymatic glycosylation of proteins is also important in development of chronic diabetic complications. The collagen molecules are thus glycosylated, glycoproteins and

proteoglycans suffer similar processes. At the level of nerves, lens, livers or other organs, glycosylation of proteins is involved in the occurrence of diabetic neuropathy, cataracts, dysmetabolic hepatopathy [Gherasim, 1998], [Kovács, 2001].

Based on these evidences, antioxidant treatment is a promising approach for complementary therapy in diabetes mellitus. Several phytotherapeutical products contain a complexity of free radical scavengers and exhibit no side effects on long term treatment.

4. Evaluation of oxidative stress and the efficacy of antioxidant treatment in diabetes mellitus

4.1. Studies on animals

Some of the plants known for their high antioxidant power are: *Allium sativa*, *Ricinus communis*, *Securinega virosa*, *Viscum album*, pomegranates, berries (including strawberries, blueberries, and raspberries), walnuts, sunflower seeds, ginger and several plants of Indian traditional medicine: *Emblica officinalis* L., *Curcuma longa* L., *Mangifera indica* L., *Momordica charantia* L., *Santalum album* L, *Swertia chirata* Buch-Ham, *Withania somnifera* and *Cassia auriculata* [Capasso et al, 2003], [Dejica, 2001], [Moshi & Mbwambo, 2003], [Pari & Latha, 2003], [Pietta, 1998], [Scartezzini & Speroni, 2000], [Varga et al., 2001].

Our studies carried out in streptozotocin diabetic rats demonstrated that treatment with the blueberry (Eridiarom®) or blueberry and sea buckthorn concentrate (Diavit®) for two months had regenerative effect on pancreatic beta cells [Crăciun et al., 2007], [Morar et al., 2004].

The therapeutic properties of blueberry are attributed to its anthocyanosides which belong to a class of substances known as plant bioflavonoids. Pharmacologically, anthocyanosides are thought to decrease vascular permeability and improve microcirculation. They are also thought to have antioxidant activity.

Diavit® is a dietary supplement with a more complex composition compared to Eridiarom®, it contains quinolizidine alkaloids, anthocyanosides, sugars, carotenoids, vitamins (C, E, PP, B₁, B₂, folic acid), minerals (K, Ca, P, S, Mg, Cl, Mn, Fe), organic acids, flavonoids, etc..

Carotenoids are best recognized for their antioxidant capacity, they are considered the most potent biological quenchers of singlet oxygen [Morar, 2003], [Paiva & Russell, 1999], [Pizzorno & Murray, 2003], [Rombi, 1998], [Slosse & Hootele, 1981], [Timberlake & Henry, 1988], [Verette, 1984], [Zeb, 2004].

The experiment was carried out on 5 groups of male Wistar rats, the first five group received Streptozotocin i.v. 4 mg/100 g body weight to induce diabetes.

The toxicity of Streptozotocin can be counteracted by desferioxamine, suggesting that oxidative reactions catalyzed by transition metals could be responsible for the toxicity of this substance. Streptozotocin, being one of the glyco-nitrosureas, could exhibit its diabetes-inducing effect also by inadequate NO release [Giugliano et al., 1995].

The first group served as a diabetic witness, the second group was treated with Siofor® ½ tablet/day/group (equivalent to 12 mg/kg), the third group received Meguan® (2 tablets of

0.5 g/group/day), the 4th group was treated with Eridiarom® (1.2 g/group/day), and the fifth group was given Diavit® (1.2 g/group/day). Healthy, normally fed rats formed the 6th, non streptozotocin-treated witness group.

After 5 days, and then each month glycaemia was measured, and after 100 days histopathologic examination (pancreas, liver, kidney, heart, muscles, eyes) were performed on 2 animals of each group, and the rest of the rats were kept under observation.

During the experiment one animal in each treated group died, and in the group treated with Siofor two animals died.

The dynamics of glycaemia increases at 7 days after the induction of subclinical diabetes (176.8 – 185.5 mg/dl \pm 3.0 – 5.6 SD) in each of the streptozotocin-treated groups compared to the initial values (103.2 – 105.3 mg/dl \pm 1.6 – 2.2 SD).

After 30 days the glycaemia is close to normal (120.1 – 127.8 mg/dl \pm 2.0 – 2.1 SD) in groups 4 and 5 treated with the phytotherapeutic products Eridiarom® and Diavit® (14.6–22.6% higher than the initial values), and it is high (151.6 mg/dl \pm 4.0 – 4.1 SD) in groups 2 and 3 treated with the antidiabetic sulphamides Siofor® and Meguan® (45.46% higher than the initial values), but lower compared to the first, witness group (164.6 mg/dl \pm 4.2 SD). After 90 days the glycaemia is practically normalized in groups 4 and 5 (102.8 – 114.3 mg/dl \pm 1.5–2.3 SD) (the final value 1.37% lower, and 9.6% higher than the initial values) and it is slightly increased in the first 3 groups (135.0 – 133.3 mg/dl \pm 3.3 – 4.0 mg/dl) (29.1–27.9% higher than the initial values).

The histopathologic examination of the pancreas (Trichromic stain) revealed a strong destructive action of streptozotocin against the endocrine and exocrine pancreas.

In the first witness group we can observe pancreatic cytonecrosis, atrophy with hypofunction and disturbing of the ratio between α and β secretory cells after administering streptozotocin. 100 days later the Langerhans islets of the rats in the first, witness group present partial regeneration and recovery of pseudolobules with β cells, general interstitial oedema, atrophy of the acini's level with the significant reducing of the cellular secretory pole.

In group 2, treated with Siofor®, 100 days after the beginning of the experiment partial recovery can be observed in the Langerhans islets, with β cells during the pseudolobules' reconstruction, and partial recovery of the exocrine secretor function by acinary and lobular hyperfunction.

In group 3 treated with Meguan®, 100 days after the initiation of the experiment regenerated Langerhans islets can be seen with α and β cells' reorganisation and the formation of sinusoidal capillary, and acinus-lobulary hyperfunction with the hypertrophy of the secretor pole in pancreatic acini.

In group 4, treated with Eridiarom®, 100 days after the beginning of the experiment, Langerhans islets with regenerated α and β cells can be observed, with increased mitotic index and complete recovery, and generalised acinus-lobulary functional hypertrophy.

In group 5, treated with Diavit®, 100 days after the initiation of the experiment, Langerhans islets with complete recovery of the cell-architectonics can be observed, specific for normal

functioning Langerhans cells, and hypertrophy with general acinus-lobulary moderate secretion.

Based on these experimental data we can conclude that the two phytotherapeutic products exhibited a powerful regenerative effect on the pancreatic cells, presenting a better efficacy compared to the widely used antidiabetic sulphamides [Crăciun et al., 2007], [Morar et al., 2004].

4.2 Studies on human subjects

In our research in humans we evaluated oxidative stress using several methods, measuring lipid peroxidation products in type 1 diabetic patients compared to non-diabetic subjects of the same age-group. We used the LPO 586 (R&D Systems) kit and two methods based on the reaction between malondialdehyde and thiobarbituric acid [Nemes-Nagy et al., 2004a], [Satoh, 1978].

After the incubation period, malondialdehyde concentration was determined by photometric dosage, in case of some methods comparing the results obtained for the samples with those for the reference series with known concentrations, previously prepared.

We revealed that oxidative stress was more intense in diabetic children (78 patients, aged 12.8 years \pm 4.2 SD) compared to healthy subjects of their age group ($P=0.0022$).

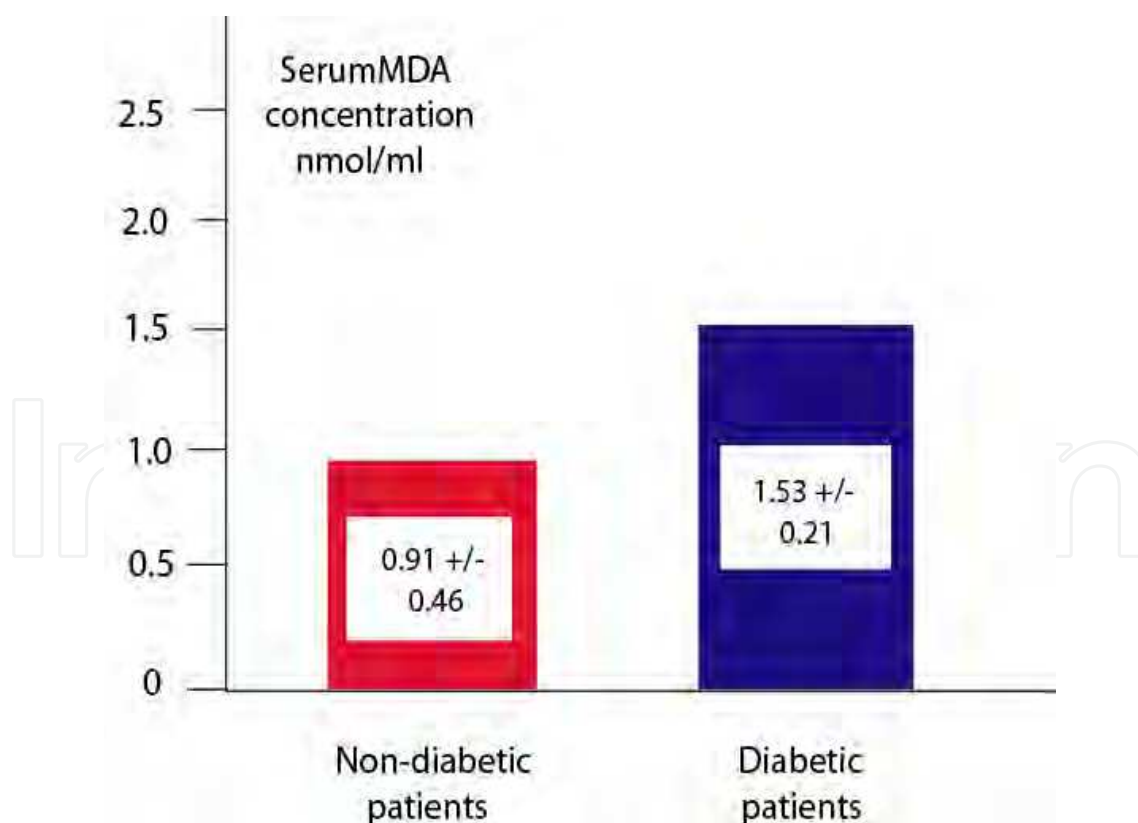


Fig. 1. Malondialdehyde concentration in diabetic and non-diabetic patients by Kei Satoh method

We obtained good correlation of the results provided by the three methods: $r = 0.8087$, $P = 0.0151$ comparing LPO-586 method with Fogelman procedure, $r = 0.6580$, $P = 0.0007$ comparing Fogelman method with the procedure described by Kei Satoh, and $r = 0.9085$, $p = 0.0007$ comparing the LPO-586 method with Kei Satoh procedure [Nemes-Nagy, 2004a].

In the diabetic children's group the average value of HbA1c was $10.0\% \pm 1.9$ (SD) (range 6.7 – 15.4 %), and the average malondialdehyde concentration was $3.7 \text{ nmol/ml} \pm 1.04$ (SD) (range 1.7 – 8.1 nmol/ml), and the MDA concentration was significantly higher ($P < 0.001$) compared to the average value of children presenting glucose intolerance ($2.6 \text{ nmol/ml} \pm 1.1$ SD).

HbA1c level highly correlated with malondialdehyde concentration ($r = 0.814$, $P < 0.05$) in the diabetic children's group (60 patients).

HbA1c was determined by chromatographic method using venous blood samples collected on EDTA- K_2 as anticoagulant.

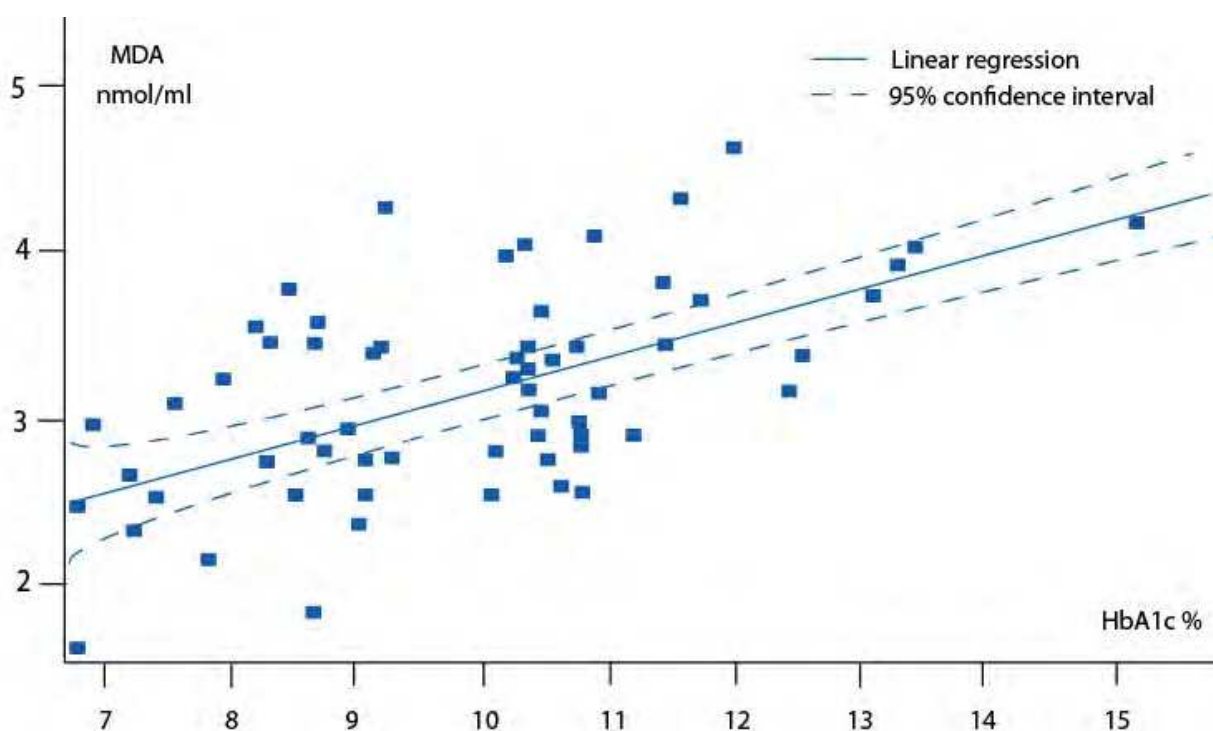


Fig. 2. Correlation between glycated hemoglobin level and serum malondialdehyde concentration in type 1 diabetic children

We also determined serum malondialdehyde concentration in three different groups of type 2 diabetic patients: subjects presenting cardiovascular (CV) diseases, patients having a high risk to develop such diseases, and those who had no other cardiovascular risk factor besides suffering from diabetes mellitus.

We obtained significant differences between the first and third ($P = 0.0015$), and the second and the third group ($P = 0.0018$) concerning malondialdehyde concentration, the patients in the third group having lower levels [Moldován, 2003] compared to those from the first two groups.

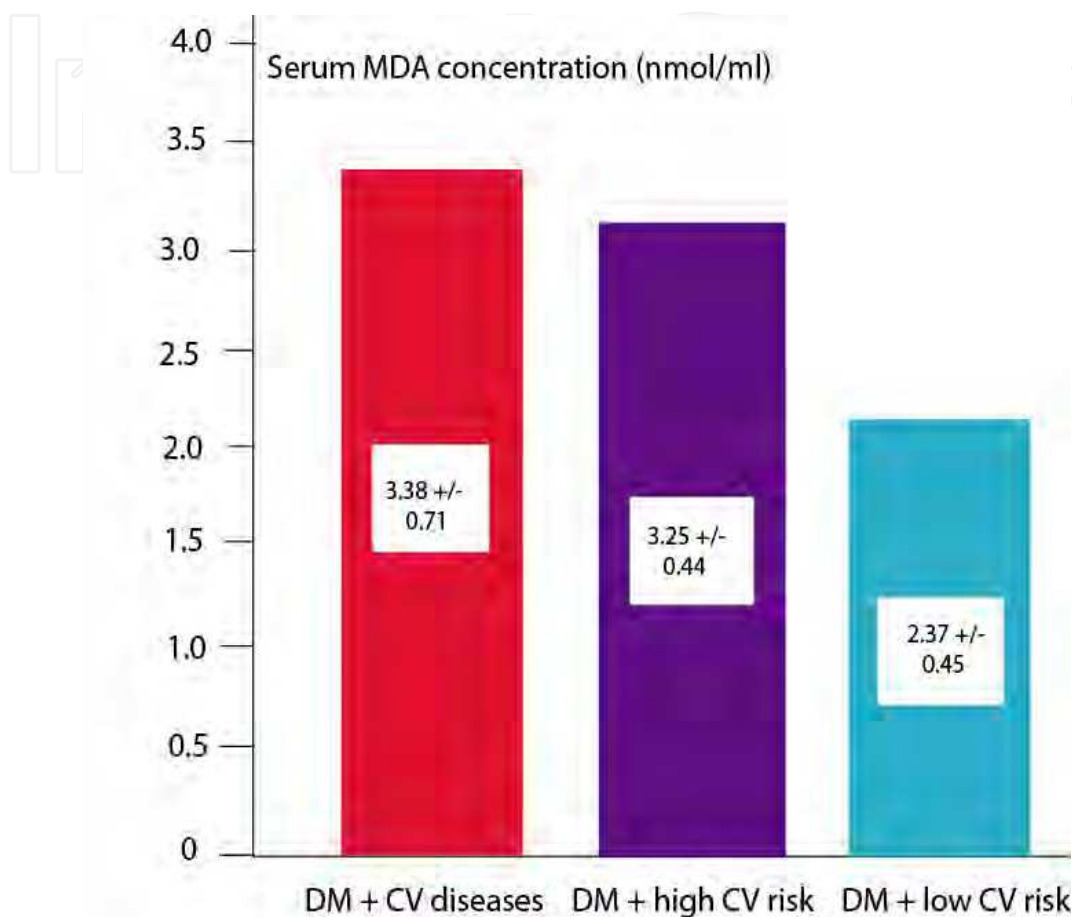


Fig. 3. Average values of serum malondialdehyde concentration in different cardiovascular risk groups of type 2 diabetic patients

We compared Cu/Zn SOD and GPX activities in diabetic children and a non-diabetic infant group of similar age. In diabetic infants we found significantly lower SOD activities ($1200.8 \text{ U/gHb} \pm 101.4 \text{ SD}$) compared to the control group ($1404.9 \text{ U/gHb} \pm 125.4 \text{ SD}$), the difference is significant ($P < 0.005$). No notable differences could be observed regarding GPX activity in the 2 studied groups of patients ($P > 0.05$).

According to the literature normal values of erythrocyte SOD are considered between 1102-1601 U/gHb. In our diabetic group only 16,7% of the patients presented values lower than normal, the minimum SOD activity value was 1014.2 U/gHb and the maximum was 1386 U/gHb.

Physiological values of GPX activity are between 27.5-73.6 U/gHb, only one of the studied diabetic children exhibited a value slightly lower than normal [Jákó et al, 2009].

Our research team demonstrated the powerful antioxidant and hypoglycemic effect of a blueberry (*Vaccinium myrtillus*) concentrate (Eridiarom®) in diabetic children (initially this product was used for treatment of diarrhoea in humans). We selected 29 infants presenting poor carbohydrate metabolic balance to participate to the study.

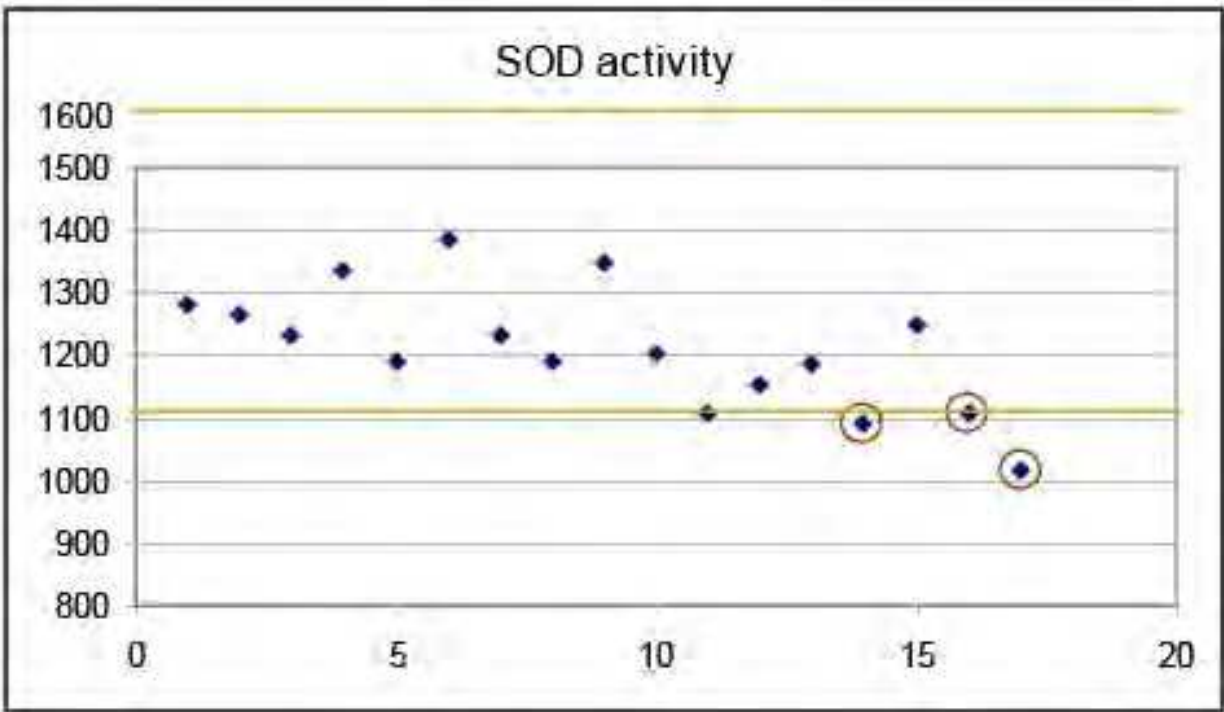


Fig. 4. Erythrocyte Cu-Zn superoxide dismutase activity in type 1 diabetic children

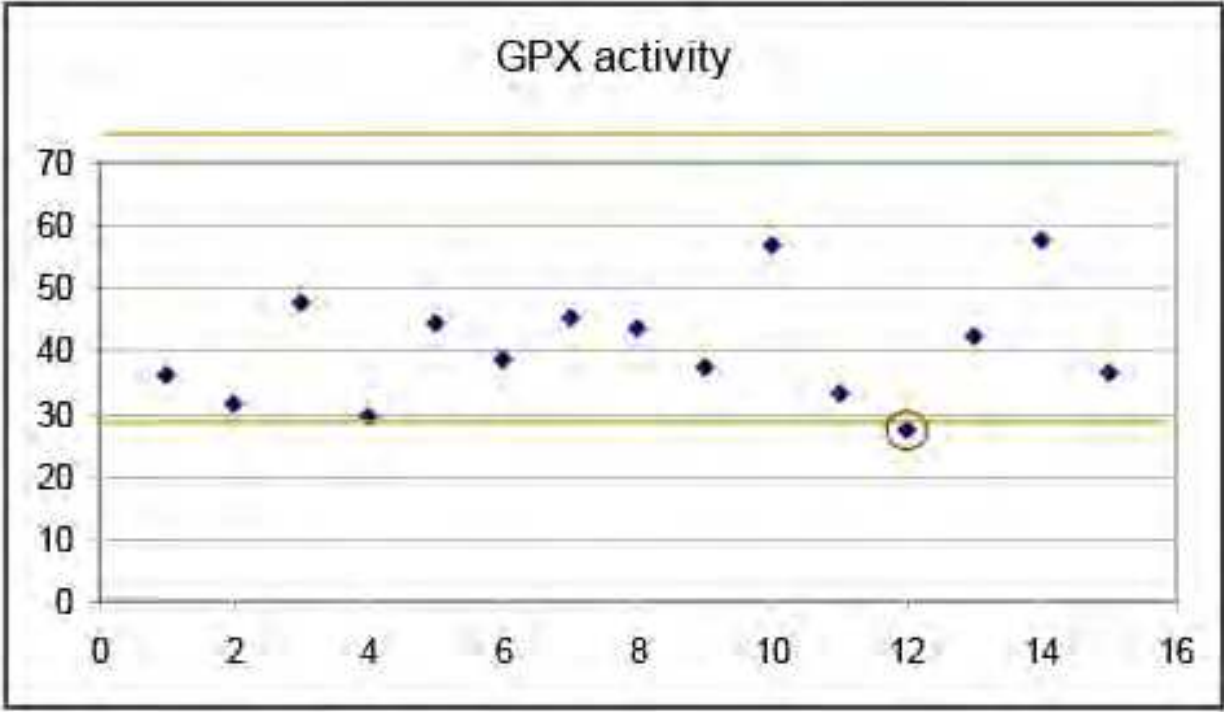


Fig. 5. Whole blood glutathione peroxidase dismutase activity in type 1 diabetic children

The average glycemic level at the beginning of the study was 179.4 mg/dl ± 39.2 (SD), after 3 months of Eridiarom® treatment the value decreased to 159.1 mg/dl +/- 40.7 (SD), the difference is significant (P<0.005).

Insulin doses (UI/kg) could be lowered in 78.6% of the patients. Initially the average dose was 0.98 UI/kg ± 0.2 (SD) and after 3 months of treatment 0.91 UI/kg ± 0.2 (SD), the difference is significant (P<0.05). This result can be explained by the regenerative effect of this phytotherapeutic product on the pancreatic beta cells, improving their insulin secretion.

We observed that the longer the treatment with this dietary supplement, better the results are.

After 3 months of Eridiarom® treatment HbA1c values presented decrease in 71.43% of the patients. Using the Student paired t test, we obtained significant differences (P<0.05) regarding the HbA1c values at the beginning of the study (9.6% ± 1.6 SD) and 8.5% ± 1.5 (SD) after 3 months of Eridiarom® treatment.

After 3 months of Eridiarom® treatment MDA concentration decreased in 92.9% of the patients, 7.1% showed practically no modification of the MDA level.

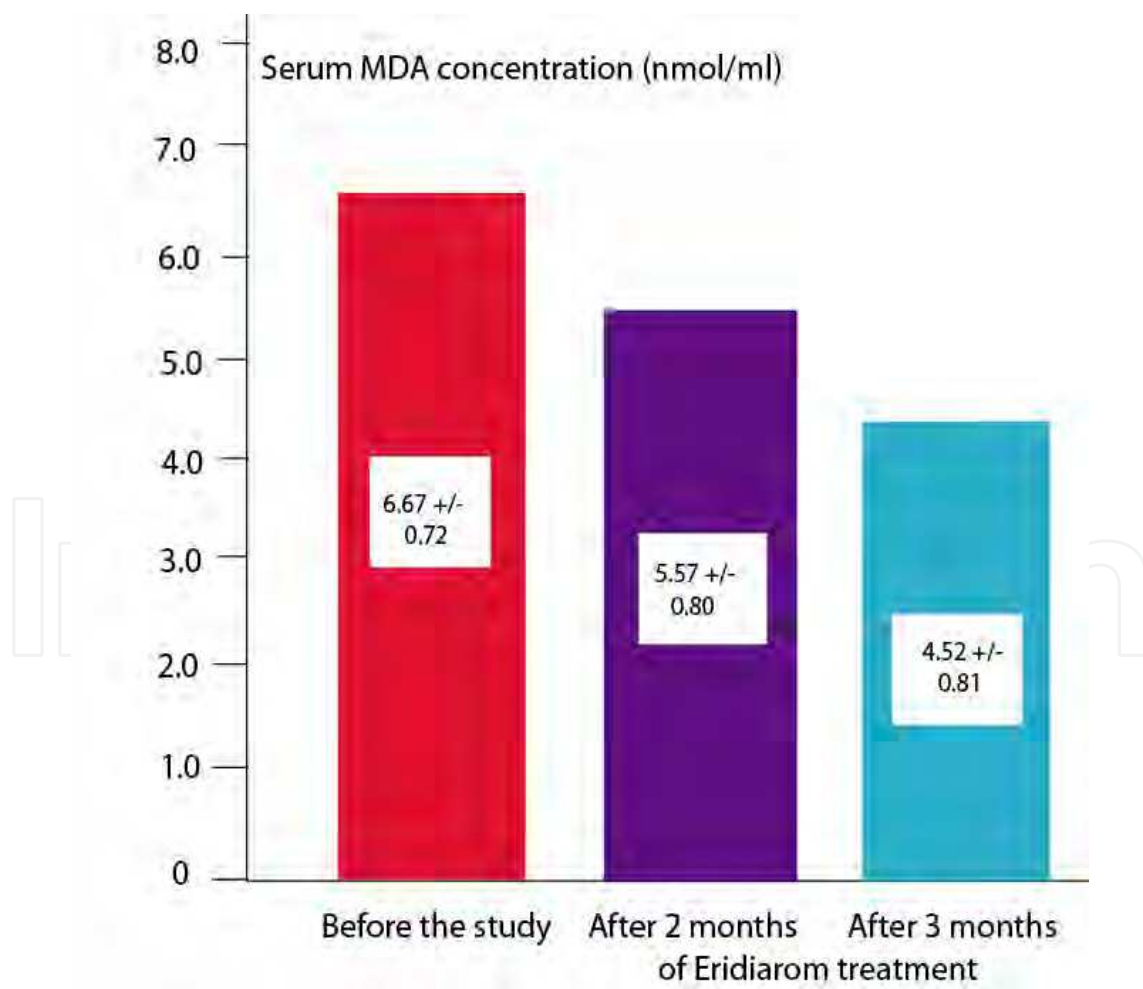


Fig. 6. Serum MDA concentration before and after Eridiarom® treatment

The average MDA concentration before the study was 6.7 nmol/ml ± 0.7 (SD), after 3 months of treatment the average value was 4.5 nmol/ml ± 0.8 (SD), the difference is significant (P<0.0001).

Prior to the study the serum magnesium and calcium concentrations were under the normal range in 43% of the patients. The average magnesium level at the beginning of the study was 1.7 mg/dl, while after 3 months of Eridiarom® treatment in case of all these patients the magnesium level turned to normal, the average value being 2.2 mg/dl; the difference is significant (P<0.0001) [Balogh-Sămărghișan et al., 2004], [Nemes-Nagy et al., 2006].

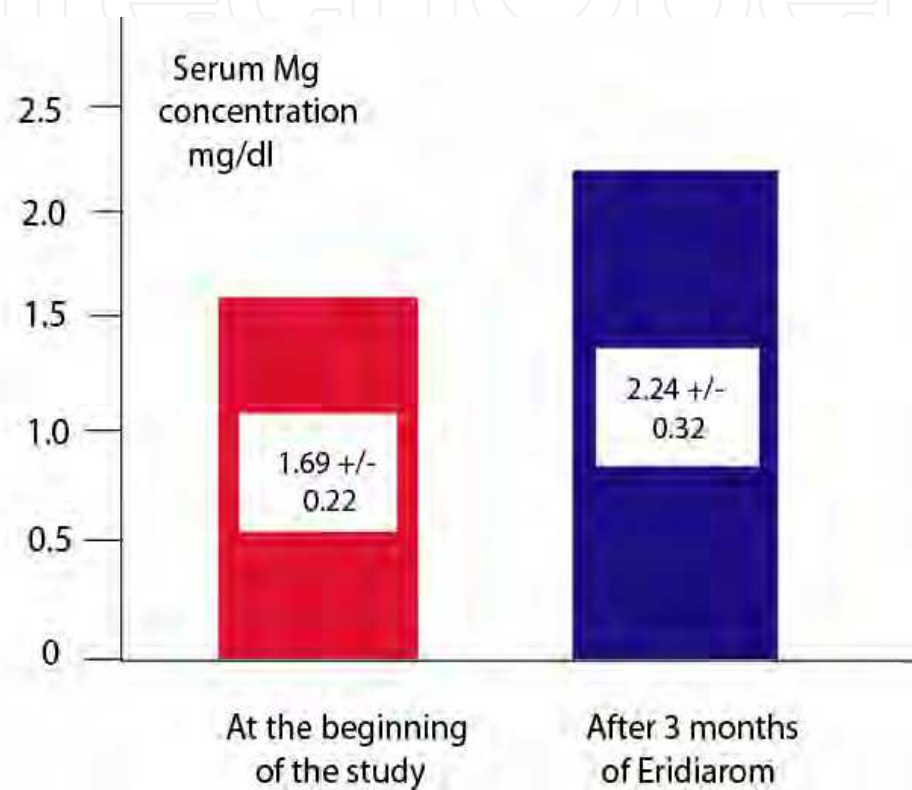


Fig. 7. Dynamics of serum magnesium concentration under blueberry containing Eridiarom® treatment

Several studies were made on the implications of magnesium in diabetes mellitus. Certain key enzymes of carbohydrate metabolism (glucokinase, hexokinase, glucose-6-phosphate dehydrogenase) and lipid metabolism (mevalonate kinase, lecithin-cholesterol acyl-transferase) are magnesium-dependent.

Insulin, along with catecholamines, has major effects on intracellular homeostasis of magnesium, being, besides vitamin D and taurine, an important magnesium-linking substance. Insulin receptors exhibit a magnesium-dependent kinase activity [Vereșiu, 2000].

Studies evaluating magnesium levels in diabetic patients compared to healthy controls showed decreases in case of diabetic subjects, and other studies have shown an increased risk for this disease in patients with magnesium deficiency. Possible correlation of Mg with chronic diabetes complications have been the subject of other studies, that have found lower values in patients with retinopathy [De Valk, 1999] and neuropathy [DeLourdes, 1998].

Effects of magnesium administration in patients with diabetes to improve glycemic control and prevention of chronic complications are conflicting and awaiting further confirmation.

A few years later we studied the effect of another dietary supplement (Diavit®), containing blueberry (*Vaccinum myrtillus*) and sea buckthorn (*Hippophae rhamnoides*) concentrate with a complex composition previously presented.

We compared glycaemic profile, glycated hemoglobin (HbA_{1c}) after 2 and 3 months of treatment, C peptide level and changes in antioxidant enzyme activity (Cu/Zn superoxide dismutase and glutathione peroxidase) after two months of treatment with the Diavit® dietary supplement versus after placebo treatment.

Values for activity of erythrocyte Cu/Zn SOD, a scavenger of superoxide radicals, were significantly higher ($P < 0.05$) in diabetic patients after two months of treatment with the concentrate (1260.9 ± 66.9 U/g Hb) compared to those obtained before treatment (1201.6 ± 105.6 U/g Hb).

There was no significant difference in GPX activity before (40.8 ± 9.2 U/g Hb) and after the study (43.9 ± 13.9 U/g Hb), only a slight, not significant increase could be observed ($P > 0.05$) [Capasso et al, 2003], [Nemes-Nagy et al., 2007, 2008, 2010], [Paglia & Valentine, 1967], [Szöcs-Molnár et al., 2006].

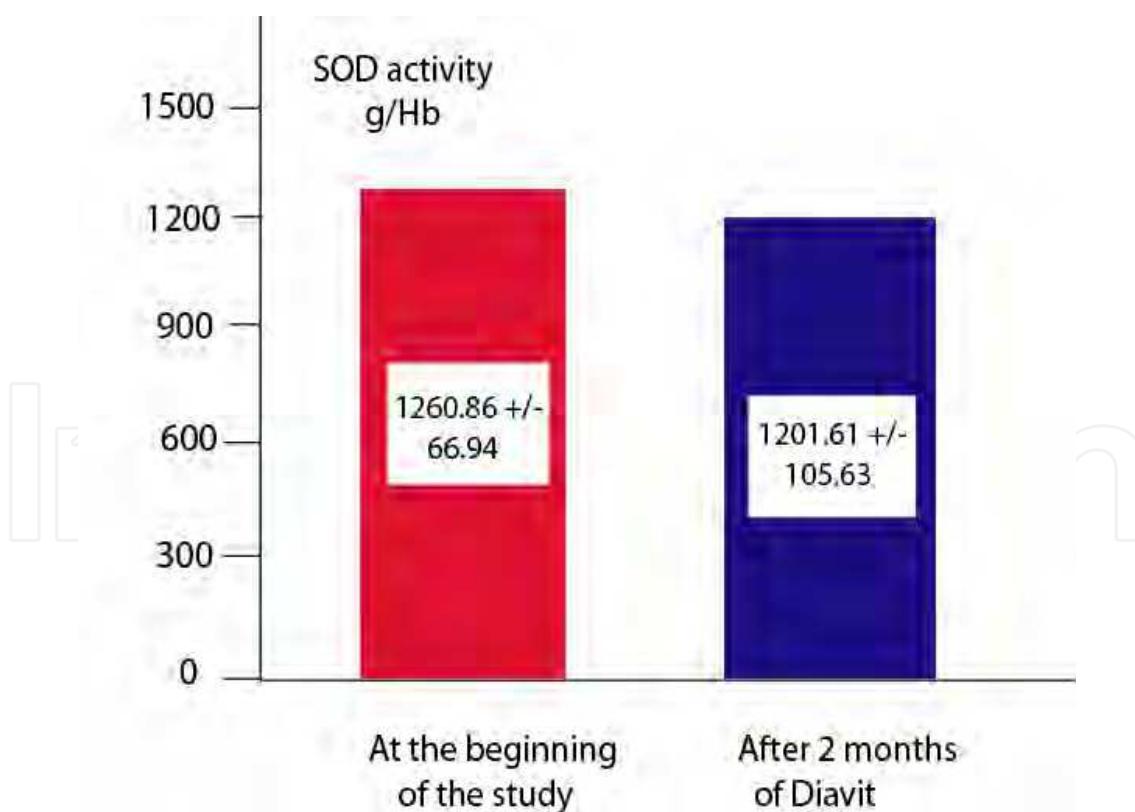


Fig. 8. Dynamics of blood SOD activity under blueberry and sea buckthorn containing Diavit® treatment

Hydrogen peroxide, formed by the reaction catalyzed by SOD, is decomposed by two enzymes: GPX and CAT. Dosage of blood catalase activity could have helped to have a better picture on the enzymatic antioxidant equipment of these diabetic patients under the treatment with this dietary supplement.

HbA_{1c} levels were significantly lower after treatment with the dietary supplement (9.2 ± 1.6 % versus the initial 10.2 ± 2.3 %; $P < 0.05$) and C-peptide average value increased significantly ($P < 0.05$) after 2 month of treatment with this dietary supplement ($0.2 \text{ ng/ml} \pm 0.1 \text{ SD}$) compared to the initial average value ($0.04 \text{ ng/ml} \pm 0.02 \text{ SD}$). Insulin requirement reduced significantly from the average of $0.96 \pm 0.27 \text{ IU/kg}$ bodyweight to $0.89 \pm 0.28 \text{ IU/kg}$ after 2 months of treatment with the product ($P < 0.05$), insulin doses were reduced in 66.7% of the patients.

Lower blood glucose and HbA_{1c} values may be due to a regenerative effect that the product has on pancreatic beta cells. Significantly higher C-peptide levels after 2 months of treatment with the extract support this hypothesis [Nemes-Nagy et al., 2008].

A scientific team in Harvard University, Howard Hughes Medical Institute, under the leadership of Prof. Douglas Melton, published their findings about the capacity of pancreatic beta cells to regenerate by self-duplication due to some latent embryonal cell remains or adult stem cells [Zhou et al., 2008], and this regenerative process might explain our results with the product studied. We suppose that better results could be obtained if the treatment with the concentrate begins soon after the diabetic disease is diagnosed, maybe because long term insulin treatment causes the atrophy of pancreatic beta-cells, similar to corticosteroid-caused hypofunction of corticosuprarenals. This hypothesis should be verified in latter studies. Based on our experimental data it seems that the longer the treatment with these dietary supplements, the better the results are.

Hyperglycemia in diabetes mellitus produces increased oxidative stress via non-enzymatic glycation, glucose autooxidation, and alteration in polyol pathway activity. This is characterized by increased lipid peroxide production and decreased antioxidative defence (e.g. inactivation of SOD by glycation) which affects the entire body. Two months using Diavit® lead to a significant increase in SOD which may have occurred as a result of its antioxidant and hypoglycemic effects. The antioxidant effect of this product might be partially due to anthocyanosides, known as scavengers of superoxide anions, inhibitors of lipid peroxidation. Lower glycaemic levels during the study might cause lower superoxide radical production and decrease the inactivation rate of this antioxidant enzyme, leading to higher SOD levels than before.

Protection of free radical scavengers might help to maintain higher levels of antioxidants under treatment with this concentrate, and several components of the product (carotenoids, vitamin E, C) show important protective role against oxidative stress. According to recent studies, it might be a link between GPX and ascorbate: intracellular vitamin C cooperates in enhancing glutathione recovery after oxidative challenge thus providing cells with enhanced survival potential, while extracellular vitamin C is recycled through a mechanism involving the simultaneous neutralization of oxidant species [Montecinos et al., 2007].

Several data suggest that oxygen metabolites are involved in the pathogenesis of autoimmune destruction of pancreatic beta cells, involving inflammatory process, and especially superoxide

radical is required for the expression of the disease. Pancreatic beta cells are particularly sensitive to superoxide mediated radical damage, having a poor antioxidant defence system. Superoxide itself or derivative radicals may be the direct cause of cell damage. Radical generation leads to breakage of cell DNA, which initiates the repair process resulting in depletion of cellular NADH + H⁺ levels, leading to an inhibition of pro-insulin synthesis and renders the cell more sensitive to radical damage because NAD is involved in the electron-transport process required for radical scavenging by the cell. Oxygen radicals are also involved in the production of the cytokines (IL-1, TNF) by the cells of the inflammatory focus that could be involved in the cell damage [Nemes-Nagy et al., 2008].

Another important component of Diavit® is the phytoestrogen called resveratrol, which is a natural polyphenolic compound found largely in the skin of red grapes, but also in blueberries.

Growing evidence suggests that resveratrol may play an important role in the prevention of many human diseases. Many of the biological actions of this polyphenol have been attributed to its antioxidant properties, it exhibits anticoagulant, vasodilator, antiinflammatory effects, inhibits oxidation of LDL-cholesterol particles, thus preventing atherosclerosis, and also increases sensitivity to insulin.

Certain studies evaluated the effect of resveratrol on intracellular reduced glutathione (GSH) and membrane sulphydryl groups in erythrocytes subjected to oxidative stress *in vitro* to test the efficacy of the antioxidant effect of resveratrol on human erythrocytes. In one of these studies subjecting erythrocytes to oxidative stress (*in vitro*) by incubating them with t-BHP (10 micromolar) caused a significant decrease in the intracellular GSH level and membrane -SH content compared with basal values.

Incubation of erythrocytes/membranes with resveratrol (1-100 micromolar final concentration) resulted in significant protection against the t-BHP-induced oxidative stress as evidenced by the increase in GSH level and membrane -SH content. It was observed that the effect of resveratrol is dose/concentration and time-dependent, it protects erythrocytes experimentally exposed to oxidative stress. Since resveratrol is naturally present in many fruits and vegetables, a diet rich in resveratrol, or dietary supplements containing this substance may provide protection against degenerative diseases and prevent diabetes complications [Pandey & Rizvi, 2010].

A new promising study on humans demonstrated the effect of regular consumption of a resveratrol supplement and the health of patients with impaired glucose tolerance. Resveratrol has been tested in relation to diabetes before, but only in animal subjects or on cell lines. Those studies have repeatedly shown promising effects on insulin secretion, insulin sensitivity and glucose tolerance, leading to the initiation of this first-of-its-kind pilot clinical study on humans [Crandall, 2010].

5. Studies on antioxidant components of medicinal teas, fruit and vegetable juices

We also determined the flavonoid content and the antioxidant capacity of several medicinal teas used by diabetic patients. We found the highest amount of flavonoids in Juglandis

folium, and the smallest amount of flavonoids was found in Phaseoli tegumen. The results were calculated in hyperoside units.

The highest concentration of malondialdehyde was found in Centauri herba (149 mmol/100g), smaller amounts were found in Myrtilli folium (146 mmol/100g), Mori folium (115 mmol/100g), Crataegi summitas (100 mmol/100g), Urticae herba (97 mmol/100g), Menthae piperitae (97 mmol/100g), Visci albae stipes (94 mmol/100g), Juglandis folium (89 mmol/100g), Millefolii flos (79 mmol/100g) and Phaseoli tegumen (73 mmol/100g). The antioxidant capacity of these plants is inversely proportional to their MDA contents, a measure of ROS-induced damage to lipids [Nemes-Nagy et al., 2004], [Lushchak Vl., 2011].

Medicinal teas	Flavonoid content (g% hyperoside)
Juglandis folium	1.79
Crataegi summitas	0.75
Menthae piperitae	0.70
Urticae herba	0.68
Mori folium	0.59
Myrtilli folium	0.31
Millefolii flos	0.24
Centauri herba	0.24
Visci albae stipes	0.18
Phaseoli tegumen	0.03

Table 1. Flavonoid content of medicinal teas

We measured, by photometric diclorphenol-indophenol method the vitamin C concentration in freshly squeezed juices and in those preserved, available in stores. As a result we found that lemon, orange and grapefruit contain the biggest ascorbate quantity, especially freshly squeezed (22.0 – 22.2 mg/dl) the same volume of juice contains higher ascorbate concentration compared to those sold in boxes (15.0 mg/dl – 21.9 mg/dl).

In case of orange juices, we compared the vitamin C content of 3 samples from juices sold in boxes from different firms (the results obtained were 20.5 mg/dl, 18.2 mg/dl and 15.0 mg/dl), so we concluded that the concentration of ascorbic acid was 7.7%, 18.0% and 32.0% lower compared to the result obtained from the freshly squeezed juices.

Freshly squeezed juices	Vitamin C content (mg/dl)
Orange	22.2
Lemon	22.2
Grapefruit	22.0
Grapes	17.2
Tomato	17.0

Table 2. Vitamin C contents of freshly squeezed juices

6. Vitamin C dynamics in human milk

We also followed the dynamics of ascorbate in human milk after juice and vitamin C tablet ingestion. The highest ascorbate level in milk was 1 hour after juice ingestion and half an hour after taking 1000 mg vitamin C containing tablets, the assimilation from natural sources being better. It is a close relationship between mothers' diet and the quality of their milk. We can recommend to consume juices one hour before breastfeeding, to offer the infant the highest amount of ascorbate [Jákó et al., 2008].

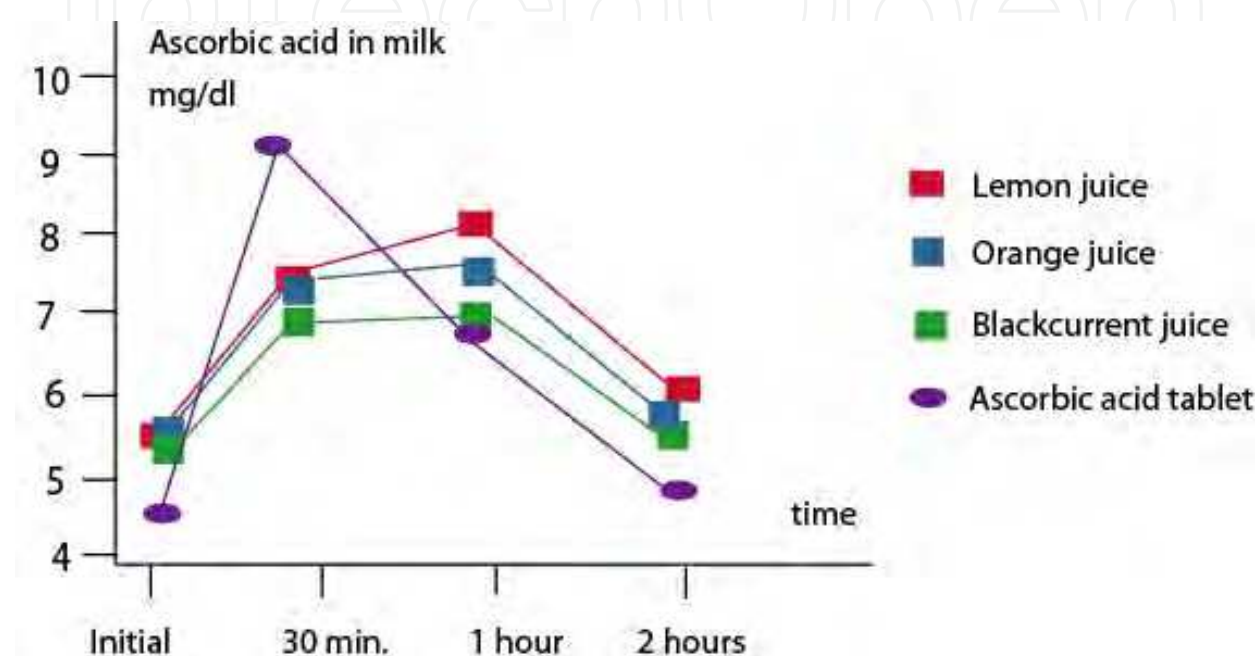


Fig. 9. The dynamics of ascorbic acid in human milk after ingestion of freshly squeezed juices and C vitamin tablets

7. Determination of carotenoid score in diabetic patients

Another study was made on adult subjects, we used Raman spectroscopy (a method that can identify carotene molecules in the skin) to determine the carotenoide score, which gives information on the antioxidant capacity of the body. Significantly lower average value was obtained in adult diabetic compared to the control non-diabetic group of similar age; the difference is significant ($P < 0.05$) [Jákó et al, 2009].

Food carotenoids are transported into the skin, protecting the tegument against ultraviolet radiation. The skin carotenoid concentration undergoes smaller variation compared to that in the blood depending on the dietary intake. Skin carotenoid determination by HPLC method using bioptic material is an invasive procedure and cannot be introduced in the every day practice. A very good correlation can be found between the serum carotenoid concentration and that in the palm skin ($r = 0.78$, $P < 0.001$) determined by Raman spectroscopy, and the biophotonic scanner operates based on this observation [Jákó et al, 2009], [Peng et al., 1995], [Smidt & Shieh, 2003]. Thus, skin carotenoid measurement appears to be a valuable biomarker of carotenoid nutritional status.

Raman scattering spectroscopy is a highly specific method for skin carotenoid determination. This method is able to discern carotenoids from other potentially interfering compounds present in the skin due to its ability to identify molecules with long conjugated double-bond structures. Raman spectroscopy involves a blue, low-energy laser light source of 470 – 490 nm, directed onto the surface of the skin, where Raman resonance light scattering events cause the carotenoids to emit a green signal at 510 – 530 nm, which is detected and quantified.

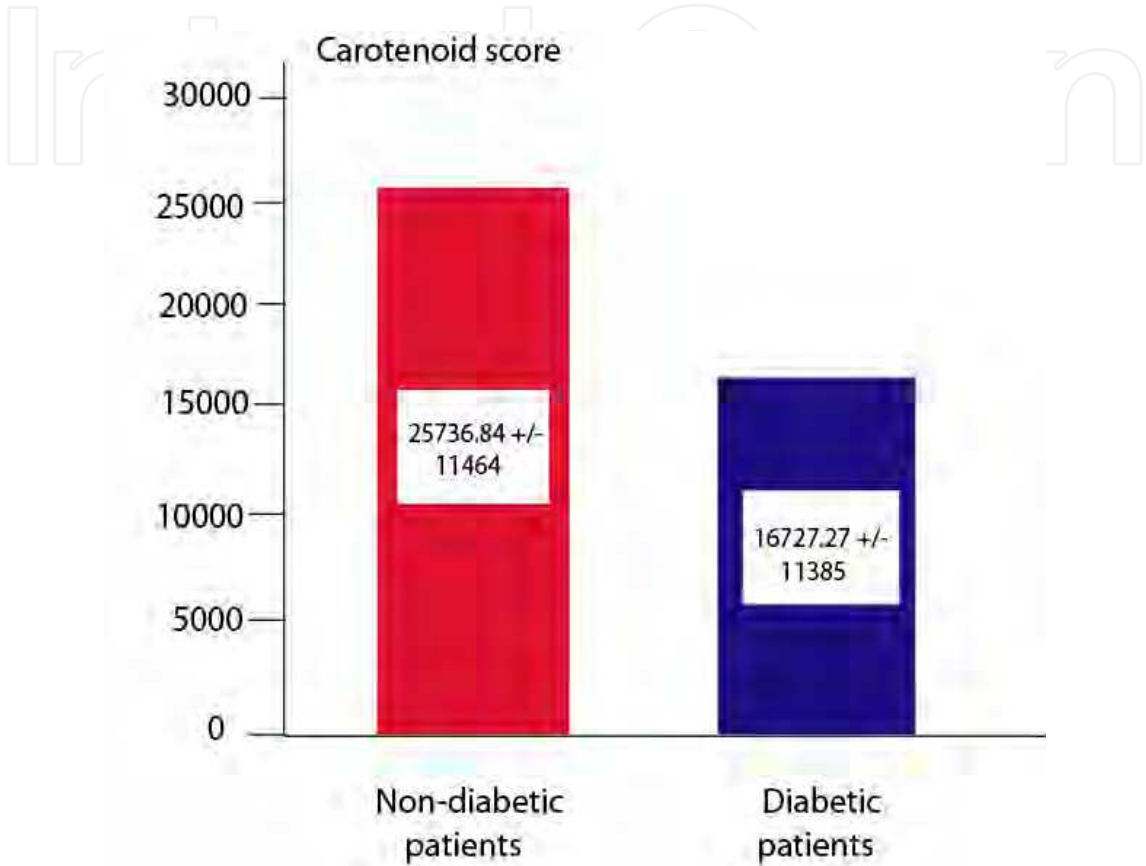


Fig. 10. Comparison of skin carotenoid score in diabetic and non-diabetic patients

Studies showed that the Raman spectroscopic method accurately reflects the presence of carotenoids in the human skin with high reproducibility. Significant differences in carotenoid concentrations were found between five different skin sites, the highest concentration being found in the palm.

An epidemiological study performed on a large number of healthy volunteers determined that the Raman spectroscopic method of measuring skin carotenoids is not really affected by gender, age or skin pigmentation.

Raman intensity measurements were positively related to the amount of fruit and vegetable intake and use of carotenoid-containing dietary supplements, and inversely related to body fat content and smoking. This study confirmed that skin carotenoids follow similar dietary and demographic patterns as in case of serum or plasma carotenoid measurements.

Before the measurement, every patient has to fill in a special questionnaire containing questions regarding its lifestyle, dietary habits and other aspects that could influence the result.



Fig. 11. Biophotonic scanner showing the carotenoid score of a non-diabetic patient



Fig. 12. Low carotenoid score of an old diabetic patient

8. Conclusion, perspectives

Regarding proper evaluation of antioxidant status in the human body, measuring lipoperoxidation products is a valuable tool because its high value positively correlates with the intensified oxidative stress. Interpretation of antioxidant enzyme activities could be sometimes difficult because it can decrease in case of intense consumption when free radical production is intensified, and in other situations even higher values can be observed due to compensatory mechanisms, being a way of adaptation to intensified oxidative stress.

We can conclude that proper nutrition including adequate fresh fruit and vegetable intake are important sources of natural antioxidants. Patients suffering from diseases like diabetes mellitus which involves high oxidative stress should take dietary supplements containing antioxidant vitamins, phytotherapeutical products and oligoelements, these could help diabetic patients to achieve a better metabolic balance and to prevent several complications of this disease.

It would be interesting to perform a placebo-controlled double-blind study on the effect of the dietary supplement Diavit®, containing blueberry and sea buckthorn concentrate, on a large group of type 2 diabetic patients followed for several years, to observe the long term effect of this phytotherapeutic product, or other, more complex dietary supplements could be used in similar studies. This could be a possibility to reduce the incidence of complications in diabetic patients, and to help them to achieve a proper metabolic balance without taking antidiabetic drugs, or at least lower the doses of their usual medication, decreasing the risk of developing side effects.

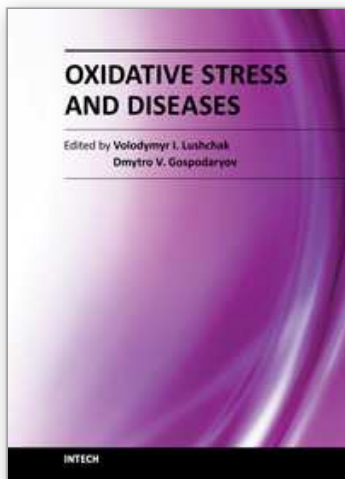
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Oxidative Stress and Diseases

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The development of hypothesis of oxidative stress in the 1980s stimulated the interest of biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with the knowledge accumulated to date on the involvement of reactive oxygen species in different pathologies in humans and animals. The chapters are organized into sections based on specific groups of pathologies such as cardiovascular diseases, diabetes, cancer, neuronal, hormonal, and systemic ones. A special section highlights potential of antioxidants to protect organisms against deleterious effects of reactive species. This book should appeal to many researchers, who should find its information useful for advancing their fields.

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