Effect of a dietary supplement containing blueberry and sea buckthorn concentrate on antioxidant capacity in type 1 diabetic children

E Nemes-Nagy¹, T Szőcs-Molnár², I Dunca², V Balogh-Sămârghițan¹, Șt Hobai¹, R Morar³, DL Pusta³, EC Crăciun⁴

¹Medical Biochemistry Department, University of Medicine and Pharmacy, Str. Gheorghe Marinescu nr. 38, cod 540139, Târgu-Mureș, Romania
²II. Clinical Hospital of Pediatrics, Târgu-Mureș, Romania
³Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania
⁴Pharmaceutical Biochemistry and Clinical Laboratory Department, University of Medicine and Pharmacy “Iuliu Hatieganu”, Cluj-Napoca, Romania

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Many studies have shown that oxidative stress plays an important role in the etiology of diabetes and its complications. New methods of treatment for prevention and control of this disease is a priority for the international scientific community.

Methods: We investigated the relationship between the glycated hemoglobin, C peptide and two antioxidant enzymes. Thirty type 1 diabetic children were treated with a blueberry and sea buckthorn concentrate for two months.

Results: After two months of administering the product to diabetic children, the erythrocyte superoxide dismutase activity was significantly higher (p<0.05). Levels of glycated hemoglobin were significantly lower (p<0.05). The activity of whole blood glutathione peroxidase was moderately increased but the difference was not statistically significant. C peptide concentration was significantly higher after treatment with this dietary supplement (p<0.05).

Conclusion: These results suggest that treatment with this dietary supplement has a beneficial effect in the treatment of type 1 diabetic children and it should be considered as a phytotherapeutic product in the fight against diabetes mellitus.

Keywords: diabetes mellitus, superoxide dismutase, glutathione peroxidase, phytotherapy, antioxidant, C peptide

Studies conducted over the last couple of years have revealed that oxidative stress plays a major role in the etiology of diabetes and its complications (2, 22). Oxidative stress is defined as an imbalance between the generation of reactive oxygen species (ROS) and...
antioxidant defense mechanisms. Free radicals inhibit insulin secretion interfering at different levels of the stimulation-secretion cycle (7, 12). There is growing evidence that ROS are involved in the autoimmune destruction of beta-cells in type 1 diabetes mellitus (19). ROS act as second messengers of interleukins (14). Hypotheses explaining the development of diabetic complications due to hyperglycemia involve the imbalance between pro-oxidants and antioxidants.

In healthy individuals, oxidative tissue damage is prevented by a defense mechanism, which includes enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase are three primary enzymes involved in direct elimination of active oxygen species (superoxide radical, hydroxyl radical and hydrogen peroxide).

Several phytotherapeutical products were tested in diabetes mellitus for their hypoglycemic effect. Many of these medicinal plants also present antioxidant properties: Allium sativa (26), Ricinus communis, Securinega virosa (11) and Cassia auriculata, used by ancient Indians (17). In a former study we demonstrated the powerful antioxidant and hypoglycemic effect of a blueberry (Vaccinium myrtillus) concentrate in diabetic children (13).

The dietary supplement used is a blueberry (Vaccinium myrtillus) and sea buckthorn (Hippophae rhamnoides) concentrate with a complex composition. It contains: quinolizidine alkaloids (23), anthocyanosides (27), sugars, carotenoids, vitamins (C, E, PP, B1, B2, folic acid), minerals (K, Ca, P, S, Mg, Cl, Mn, Fe), organic acids, flavonoids, etc. (9, 21, 28). 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 (18, 25). Carotenoids are best recognized for their antioxidant capacity. In fact, carotenoids are considered the most potent biological quenchers of singlet oxygen (16).

Studies carried out in streptozotocin diabetic rats demonstrated that treatment with a blueberry or blueberry and sea buckthorn concentrate for two months had a histologically visible regenerative effect on pancreatic beta cells. The glycemia also decreased during the treatment (10).

This study was designed to evaluate activities of two antioxidant enzymes (SOD and GPx) in patients suffering from type 1 diabetes, and also, to examine the relationship between these parameters and glycemic control after two months of a blueberry and sea buckthorn concentrate administration.

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**Abbreviations**

GPx, glutathione peroxidase; Hb, hemoglobin; HbA1c, glycated hemoglobin; ROS, reactive oxygen species; SOD, superoxide dismutase; U, unit; WBC, white blood cell.
Materials and Methods

Subjects

The study included 30 children ages 8 to 17 suffering from type 1 diabetes the average of their age was 12.6±3.65 (SD) years. It was conducted at the II. Clinical Hospital of Pediatrics in Târgu Mureș, Romania. Informed parental consent was obtained. The County Clinical Hospital in Târgu Mureș approved all procedures pertaining to the study.

The study was carried out between January and June 2006. The starting date was not the same for all patients. We selected patients presenting high HbA1c values in spite of intensive insulin treatment, only 3 children in the study group had HbA1c under 7.5% at the beginning of the study. In the first two months patients were given a placebo. In the next two months the dietary supplement was administered, 3×1 comprimates/day, before meals. A washout period was not considered necessary. Intensive insulin treatment (4–5 injections/day) continued during the study, using rapid-acting insulin three times per day and long-term insulin in the evening (some children were given an extra dose of rapid-acting insulin at 2 o’clock in the night). Insulin doses were adjusted depending on carefully monitored glycemic levels to prevent dangerous hypoglycemia while taking this dietary supplement.

We compared values for glycemia (measured in the morning, before the first dose of insulin), glycated hemoglobin (HbA1c), C peptide level and changes in antioxidant enzyme activity after two months of treatment with the dietary supplement versus after placebo treatment (n=28 samples).

Assay of superoxide dismutase (SOD) activity

Venous blood samples were collected using Vacutainer tubes containing K$_3$EDTA. Whole blood (0.5 mL) was centrifuged for 10 minutes at 3000 rpm and plasma was removed. Erythrocytes were washed three times with 0.9% NaCl solution, lysed by adding up to 2.0 mL cold redistilled water followed by vigorous vortex-mixing and incubated at +4 °C for 15 minutes. Lysate was diluted with 0.01 mol/L phosphate buffer pH 7.0 to achieve an enzyme inhibition between 30 and 60%.

Cu/Zn SOD activity was measured with RANSOD kit (cat. No. SD125; RANDOX Labs., UK). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-indophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. The activity was measured at 37 °C on a Cobas Mira Plus (Roche), and absorbancy was monitored at 505 nm. The unit (U) of activity is defined as the amount of enzyme that inhibits the rate of the formazan dye formation by 50%.

Assay of glutathione peroxidase (GPx) activity

Venous blood samples were collected in heparinised tubes. GPx activity was determined with RANSEL kit (Cat. No. RS505, Randox Labs., UK) at 37 °C on a
Cobas Mira Plus (Roche) at 340 nm. This assay, based on the method of Paglia and Valentine (15), requires cumene hydroperoxide as a substrate. Before analysis the samples were diluted 40-fold by adding diluting agent (to convert the glutathione to reduced form) and after double strength Drabkin’s reagent (to inhibit the positive interference of peroxidases present in human blood).

The activities of SOD and of GPx were expressed in U/L and U/gHb.

Hemoglobin concentration was determined on the Sysmex KX-21N analyzer (Kobe, Japan) from K$_3$EDTA blood samples using a non-cyanide hemoglobin analysis method that rapidly converts hemoglobin into methemoglobin. These non-cyanide methods usually contain sodium-laurylsulfate in their reagent (5). In the first step, blood is aspirated into a sample rotor valve from where 6 µL of blood are transferred to a white blood cell (WBC) transducer chamber along with 1.994 mL of diluent. At the same time 1 mL of lyse is added to prepare a 1:500 dilution of this sample. During a 10-second phase red blood cells are hemolysed and platelets shrink while white blood cell membranes remain intact. Hemoglobin (Hb) is converted into methemoglobin.

From the WBC transducer chamber, approximately 1 mL of the diluted/hemolyzed sample is transferred to a Hb flow cell where Hb absorbance is read at a wavelength of 555 nm using a light emitting diode. Zero absorbance is set with diluent.

Fresh capillary blood was obtained to measure glycemias using Accu-Check® Active, Roche (1), which uses a colorimetric method based on the reaction between glucose and oxidoreductase.

Assay of glycedated hemoglobin was performed by an immunoturbidimetric method (reagents from Thermo Electron Corporation) on the Konelab analyzer (6), using hemolysed blood samples. HbA1c in the sample reacts with anti-HbA1c antibodies present in Reagent A to form a soluble antigen-antibody complex. Polyhaptenes in Reagent B react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex measured by this method.

C peptide was determined using a commercial C peptide kit (ROCHE) on an Elecsys 2010 (ROCHE, Switzerland) analyzer using a method based on electrochemiluminescence. During the first incubation period 20 µL of the sample (serum) is mixed with monoclonal anti-C peptide antibodies specifically marked with biotine and monoclonal anti-C peptide antibodies specifically marked with ruthenium, forming a sandwich. During the second incubation period microparticles binding streptavidine are placed into the reaction cuvette. The immune-complex is fixed to the solid phase through a streptavidine-biotin bond. The next step takes place in the measuring cell, the microparticles are maintained at the level of the electrodes. The elimination of the free fraction is processed by passing through the ProCell. A difference of potential is applied to the electrode and produces luminescence measurable with a photomultiplier. Results are obtained using a calibration curve. Normal range of C peptide is between 1.1–4.4 ng/mL for adults (3). Pediatric reference values are: 0–9 years: 0.0–3.3 ng/mL, 10–16 years: 0.4–3.3 ng/mL, >16 years: 1.1–5.0 ng/mL (20). The method has a good reproducibility, CV (coefficient of variation) is 2.3% for human serum samples.
Data analysis

We performed statistical analyses using the GraphPad InStat program, paired t-test, occasionally with Welch-correction (in case of significant difference between standard deviations). All data passed Kolmogorov and Smirnov normality test for Gaussian distribution. Numeric results show mean values followed by standard deviation (SD).

Results

Values for activity of erythrocyte Cu/Zn SOD (Fig. 1), a scavenger of superoxide radicals, were significantly higher (p<0.05) in diabetic patients after two months of treatment with the concentrate (1260.86±66.94 U/g Hb) compared to those obtained before treatment (1201.61±105.63 U/g Hb) (4).

We performed a reproducibility test on erythrocyte SOD assay. The mean relative percentage error is 0.71%, the coefficient of variation is 0.88%, so the method is highly reproducible.

The activity of whole blood GPx (Fig. 2) was also moderately increased after two months of treatment with the product but the difference is not statistically significant (43.93±13.85 U/g Hb versus 40.80±9.24 U/g Hb; p>0.05).

Reproducibility test for whole blood GPX assay shows a mean relative percentage error of 2.29%, and the coefficient of variation is 2.71%.

The correlation between activities of antioxidant enzymes (SOD and GPx) and levels of HbA1C was also studied but did not correlate well according to the correlation coefficient.

HbA1C levels were significantly lower (Fig. 3) after treatment with the dietary supplement (9.22±1.6% versus 10.24±3.44%; p<0.05). Glycemia showed significant improvement under treatment with an average value of 10.68±2.60 mmol/L before the extract and 9.17±3.08 mmol/L after 2 months of treatment; p<0.05. Hypoglycemic episodes became more frequent under treatment with the product. Patients were informed about this possibility and proper adjustment of insulin doses prevented any problems that could occur in case of hypoglycemia. Insulin requirement reduced significantly from the average of 0.96±0.27 IU/kg bodyweight to 0.89±0.28 IU/kg after 2 months of treatment with the product (p<0.05), insulin doses were reduced in 66.7% of the patients.
**Fig. 1.** SOD activity before and after two months of treatment with the dietary supplement ($p<0.05$)

**Fig. 2.** Variation of GPX activity during 2 months of treatment with the product ($p>0.05$)
In a group of patients (n=10) we followed C-peptide concentration during the study, which shows the level of endogenous insulin production. Before taking the product, these patients had a mean C-peptide level of 0.044±0.02 (SD) ng/mL, and the average value increased to 0.198±0.14 (SD) after 2 month of treatment with this dietary supplement. The difference is statistically significant, p<0.05. Comparing our results with the reference range of C peptide for children of their age (0.4–3.3 ng/mL), some of the patients had C peptide values close to the lower limit (e.g. 0.324 ng/mL) or even inside the interval (e.g. 0.411 ng/mL), while all patients presented values under 0.08 ng/mL before taking the concentrate. Albuminuria was present in 2 subjects in the study group (30 mg/dL), the situation was unchanged after treatment with the product.

Discussion

Lower blood glucose and HbA1c 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.

A scientific team in Harvard University, Howard Hughes Medical Institute, under the leadership of Prof. Douglas Melton, published in 2004 (Nature, vol. 429) their findings about the capacity of pancreatic beta cells to regenerate by self-duplication due
to some latent embrional cell remains or adult stem cells, 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 later studies.

Regenerative effect of this product on pancreatic beta cells was demonstrated experimentally on animals, where regeneration process was supported by histological examinations: after 2 months of treatment a partial recovery was shown, and the aspect was very close to normal after 3 months of therapy with this extract. Rats in control group not taking the product showed disorganized beta pancreatic islet structure also 3 months after streptozotocin induced diabetes (4, 10). Based on experimental data it seams that the longer the treatment with this dietary supplement, the better the results are.

Several data suggest that oxygen metabolits are involved in the pathogenesis of autoimmune distruction of pancreatic beta cells, involving inflammatory process, and especially superoxid 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 NAD levels, leading to an inhibition of pro-insulin syntheis 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 (14).

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 this dietary supplement 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 superoxid 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, vitamins 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 (8).
A possible limitation of our study is that our patients were given placebo in wintertime, and we started treatment with the dietary supplement in early spring, when their diet might have been slightly changed, but we tried to avoid this problem by evaluating lifestyle and diet of the patients using a complex questionnaire filled in at the beginning of the study and the pediatric diabetologist of our team controlled the patients’ diet during the study period maintaining it relatively unchanged in different seasons.

Conclusions

Based on data presented, we conclude that two months of treatment with this dietary supplement increased antioxidant activity in type 1 diabetic children. This is seen as a statistically significant increase in SOD, and a smaller, not significant rise in GPx level. It would be interesting to determine catalase activity (which uses the same substrate as GPX) and the variation of plasma total antioxidant capacity during the treatment.

The dietary supplement tested is a well-tolerated, non-toxic product. It might be administered on a long term basis to diabetic patients. Hypoglycemia may occur but it can be controlled by proper adjustment of insulin doses (24). Based on product safety, its powerful antioxidant and hypoglycemic effect, we conclude that this dietary supplement containing blueberry and sea buckthorn concentrate deserves to be one of the phytotherapeutics in the fight against diabetes mellitus.

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